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Developmental-Stage-Specific Expression of the hsp70 Gene Family during Differentiation of the Mammalian Male Germ Line

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Mouse somatic tissues contain low levels of transcripts homologous to the heat shock-inducible and cognate members of the heat shock protein 70 (hsp70) gene family. An abundant, unique sized hsp70 mRNA of 2.7 kilobases (kb) is present in testes in the absence of exogenous stress. Its expression is restricted to germ cells and is developmentally regulated. The 2.7-kb transcript first appears during the haploid phase of spermatogenesis and is stable throughout the morphogenic stages of spermiogenesis. A 2.7-kb hsp70 mRNA is present in rat and human testes. These observations suggest that a member of the hsp70 gene family plays a role in the development of the mammalian male germ cell lineage.

Procaryotic and eucaryotic organisms respond to environmental stresses such as mildly elevated temperatures by synthesizing a small set of highly conserved cellular proteins called heat shock proteins (HSP) (1, 11, 30, 36–38). The genes coding for HSP are characterized by a high level of DNA sequence conservation not only in transcribed regions but also in certain regulatory sequences (3, 18, 19, 31). Evolutionary conservation of the stress response at physiological and molecular levels suggests that HSP may have basic functions in the maintenance of cellular homeostasis (11, 22, 30, 32, 36–38).

HSP are also synthesized during normal development and differentiation. Members of the *hsp70* gene family have been shown to be expressed during early development of such diverse organisms as frogs (7), mice (5, 6), and *Drosophila* spp. (14, 17). HSP are believed to be among the first products resulting from zygotic gene activity in early mouse embryos (5). Such spontaneous or developmentally induced expression can further be detected in ectoderm of day 8 mouse embryos, as well as in differentiated embryonal carcinoma cells (6, 28, 42).

HSP have also been shown to be expressed in the germ line of lower eucaryotes. The *hsp26* gene is expressed abundantly during oogenesis in *Drosophila* spp. around follicular growth stage 10 (35, 45). *hsp26* is also expressed in the male germ line in *Drosophila* spp., primarily in spermatocytes, with little or no expression in spermatogonial stem cells (16). A conserved function for HSP in germ cell development was suggested in recent studies by Kurtz et al. (21). *hsp26*, *hsp84*, and two *hsp70*-related proteins were shown to exhibit a pattern of expression during acrospore formation of yeasts that is virtually identical to that observed during *Drosophila* oogenesis.

The sensitivity of mammalian male germ cells to increased temperature has been observed since the time of Hippocrates (cited in reference 34). The cellular and molecular basis for this sensitivity is unknown. However, it is the germinal compartment of the testis which is most readily destroyed by external stresses (27, 44). The observations of developmentally regulated HSP expression in germinal and meiotic cells in lower eucaryotes, combined with the known sensitivity of male mammalian germ cells to elevated temperatures, prompted our investigation into the pattern of expression of the *hsp70* gene family during mammalian gametogenesis. Here we report the abundant expression of a novel *hsp70*-related transcript whose presence in testes is restricted to germ cells and is correlated with the onset of the haploid stage of germ cell differentiation.

MATERIALS AND METHODS

Probes and tissues. Male Swiss Webster mice were used as a source of normal mouse tissues and normal testes. Testes were also obtained from F_1 male mice of C57BL/6J × DBA matings and from Sprague Dawley adult male rats. Portions of human testes were obtained from pathological specimens removed as part of standard surgical procedures. Such specimens were examined histologically to assess the state of the spermatogenic process. For developmental studies, testes were collected from animals at days 7 and 17 of neonatal life. W series mutant mice (26) and their homozygous progeny were used in the studies on germ cell-deficient testes. Normal testicular cells in different stages of spermatogenesis were separated by sedimentation at unit gravity as described by Wolgemuth et al. (43).

The following probes were used: (i) a 1.3-kilobase (kb) *Hin*dIII-*Eco*RI insert from plasmid pMHS213 [The plasmid pMHS213, containing a 1.3-kb *hsp68* cDNA insert into the *Kpn*I site of vector pDPL13, was a gift of L. Moran (23)]; and (ii) pMET1, a plasmid containing two copies of mouse metallothionein-1 cDNA (15; a gift from R. Palmiter).

Cell culture and heat shock conditions. Mouse L cells were grown in Dulbecco minimal essential medium with 10% fetal calf serum at 37°C with 5% CO_2. The heat shock treatment was a modification of that described by Lowe and Moran (23). L cells were heat shocked at 43°C for 90 min and allowed to recover for 2 h at 37°C. Cells were lysed directly on culture plates, and RNA was isolated and analyzed as described below.

Analysis of mRNA. RNA was isolated from the different tissues and separated testicular cell populations by the LiCl precipitation method described by Cathala et al. (9). Poly(A)$^+$ RNA was selected through one cycle of oligo(dT) cellulose chromatography (2). RNA that is not retained by oligo(dT) is termed flowthrough RNA. RNA samples were

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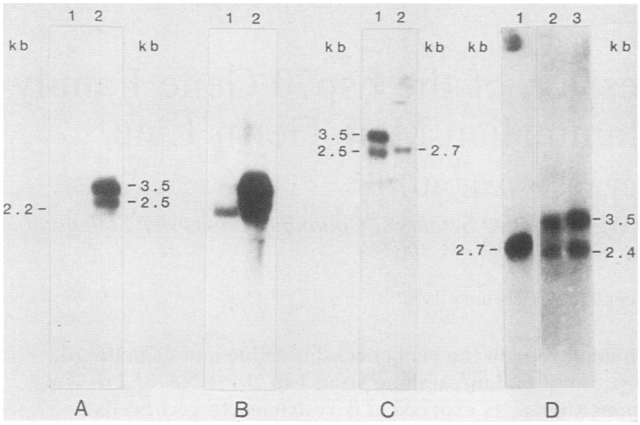

FIG. 1. *hsp70* gene transcripts in mouse L cells and normal mouse tissues. RNAs were electrophoresed in denaturing 0.8% agarose–2.2 M formaldehyde gels and processed for Northern blot hybridization analysis with a ^{32}P-labeled 1.3-kb *Hin*dIII-*Eco*RI insert from pMHS213. (A) Lane 1, 20 μg of total RNA from mouse L cells; lane 2, 20 μg of total RNA from heat-shocked mouse L cells. (B) Panel A overexposed. (C) Lane 1, 20 μg of total RNA from heat-shocked mouse L cells; lane 2, 30 μg of total RNA from mature Swiss Webster mouse testes. (D) Lane 1, 20 μg of total RNA from mature Swiss Webster mouse testes; lane 2, 10 μg of poly(A)$^+$ RNA from mouse brain; lane 3, 10 μg of poly(A)$^+$ RNA from mouse liver. Exposure times (days): panel A, 1; panel B, 8; panel C, 2; panel D, 5.

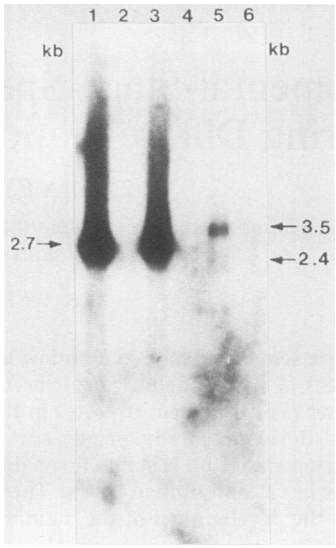

FIG. 2. Detection of *hsp70* transcripts in mutant mouse germinal tissues. RNAs [poly(A)$^+$ and flowthrough] isolated from testes of mature Swiss Webster mice were analyzed on 1.5% agarose–formaldehyde gel and hybridized with a ^{32}P-labeled 1.3-kb *Hin*dIII-*Eco*RI insert from pMHS213 as described in Materials and Methods. Lanes: 1 and 2, adult Swiss Webster mouse RNA; 3 and 4, wild (+/+) littermates of the progeny of a W/+ × W^v/+ mating; 5 and 6, homozygous W^v/W mice. Lanes 2, 4, and 6 each contained 20 μg of flowthrough RNA; lanes 1, 3, and 5 contained 5 μg of poly(A)$^+$ RNA. Exposure time, 7 days.

electrophoresed on denaturing 0.8 or 1.5% agarose–2.2 M formaldehyde gels. Gels were blotted onto nitrocellulose paper overnight as described by Maniatis et al. (24) and baked for 3 h at 80°C. Probes were labeled by nick translation with ^{32}P-labeled deoxynucleoside triphosphates (41). Hybridization was essentially as described by Wahl et al. (39), at high stringency in the presence of 10% dextran sulfate–50% formamide–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After hybridization, filters were washed sequentially for 20 min each in 2× SSC–0.1% sodium dodecyl sulfate (SDS), 1× SSC–0.1% SDS, 0.1× SSC–0.1% SDS, and 0.1× SSC alone, all at 65°C.

RESULTS

Detection of a unique *hsp70* transcript in testis. RNA was isolated from three sources for these initial studies: (i) heat-shocked and non-heat-shocked mouse L cells; (ii) somatic tissues; and (iii) adult mouse testis. The RNAs were analyzed by Northern blot hybridization with as a probe, the insert of clone pMHS213, a cDNA for the heat-inducible mouse *hsp68* gene. This gene is a member of the *hsp70* family, which in mice includes at least 3 to 5 genes (23). In heat-shocked mouse L cells, this probe recognized two induced transcripts: a major transcript migrating at ~3.5 kb and a second, less abundant transcript migrating at ~2.5 kb (Fig. 1A, lane 2; also, reference 23). In nonstressed mouse L cells, the probe detected a very faint transcript migrating at ~2.2 kb, which was also present in the induced cell line (Fig. 1A and B, lanes 1 and 2).

In testis, an abundant transcript of ~2.7 kb (2.7-kb T-HSP70) was observed (Fig. 1C, lane 2, and D, lane 1). This transcript did not comigrate with the transcripts found in heat-shocked mouse L cells. The testicular transcript is abundant, since it was readily detected in 20 μg of total RNA, which represents ~0.5 to 1 μg of poly (A)$^+$ RNA. This probe also detected transcripts in mouse somatic tissues, including liver and brain (Fig. 1D, lanes 2 and 3). These transcripts, ~3.5 and ~2.4 kb long, were detected in other somatic tissues such as kidney, prostate, placenta, and thymus (data not shown). These transcripts were distinct in size from the ~2.7-kb testicular transcript (Fig. 1D, lane 1 versus lanes 2 and 3). The two somatic transcripts in liver and brain were also clearly less abundant than the testicular transcript, since 10 μg of poly (A)$^+$ RNA was required to produce a readily detectable signal.

Cellular origin of the unique 2.7-kb transcript. The abundant 2.7-kb T-HSP70 transcript is likely to be of germ cell origin, since germ cells comprise more than 90% of the cells in adult mouse testes (25). RNAs were prepared from testes of a germ cell-deficient mutant strain of mice of the W series genotype (26) to examine this possibility. Gonads from mice homozygous for the W alleles (W^v/W) are virtually devoid of germ cells (10). Histological examination of W^v/W testes reveals a normal interstitium and well-formed seminiferous tubules lined by Sertoli cells but few or no identifiable germ cells (10).

RNA was isolated from testes of sexually mature siblings of a W^v/+ × W/+ mating. Homozygous animals can be distinguished from their wild-type littermates by (i) lack of coat pigmentation and (ii) black eyes (10). Poly(A)$^+$ RNA from the wild-type littermates (Fig. 2, lane 3) and Swiss Webster mice (lane 1) contained the 2.7-kb T-HSP70 transcript. In contrast, no 2.7-kb transcripts were detected in germ cell-deficient W^v/W testes (lane 5). The two transcripts characteristic of somatic tissues were present, with the 3.5-kb species being the most abundant. These results suggest that expression of the 2.7-kb T-HSP70 transcript depends on the presence of germ cells since all of the somatic

cell types are present in the testes of the homozygous W^v/W animals.

The 2.7-kb T-HSP70 transcript is developmentally regulated. We took advantage of the biological features of testicular development in neonatal mice to determine whether the appearance of the 2.7-kb T-HSP70 transcript is correlated to different stages of germ cell development. Testes from day 7 to 8 neonates contain primitive type A spermatogonia as well as the more advanced type A and B spermatogonia (4, 29). Premeiotic DNA synthesis occurs at neonatal day 9 to 10, and the cells then enter meiotic prophase. Cells in all stages of meiotic prophase, as well as the first round of cells that have completed the meiotic divisions, are found in day 17 mice. In sexually mature animals, virtually the complete cell lineage, from primitive type A spermatogonia to mature sperm, is present. Testes at each of these developmental ages contain the typical somatic cell components, including Leydig and Sertoli cells.

RNA was isolated from testes at the stages described above and analyzed by Northern blot hybridization analysis with the insert from pMHS213 (Fig. 3). Neonatal day 7 to 8 and day 17 testes contained the typical somatic transcripts, with the 3.5-kb transcript being most abundant (Fig. 3, lanes 4 and 6). Only mature testes contained the 2.7-kb T-HSP70 transcript (lane 2). This result suggests that this transcript is not present, or is present in very low amounts, in mitotic or early meiotic germ cells.

To determine more precisely which spermatogenic cells contained the 2.7-kb T-HSP70 transcript, we purified populations of meiotic prophase spermatocytes (predominantly in the pachytene stage of meiosis), postmeiotic early spermatids, and a mixture of residual bodies and cytoplasmic fragments from elongating spermatids (43). RNAs were isolated from the various cell types and analyzed by Northern blot hybridization with the pMHS213 insert as a probe (Fig. 4).

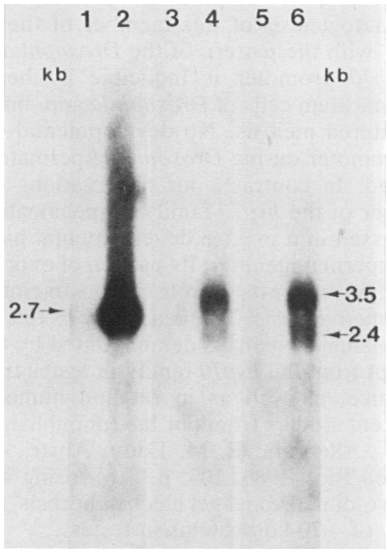

FIG. 3. Developmental-stage-specific expression of the *hsp70* transcript in testes. RNAs were isolated from testes of adult mice (lanes 1 and 2), day 17 postnatal mice (lanes 3 and 4), and day 7 neonatal animals (lane 5 and 6), as described in the legend to Fig. 2. Lanes 1, 3, and 5 each contained 20 μg of flowthrough RNAs; lanes 2, 4, and 6 each contained 5μg of poly(A)$^+$ RNA. Exposure time, 3 days.

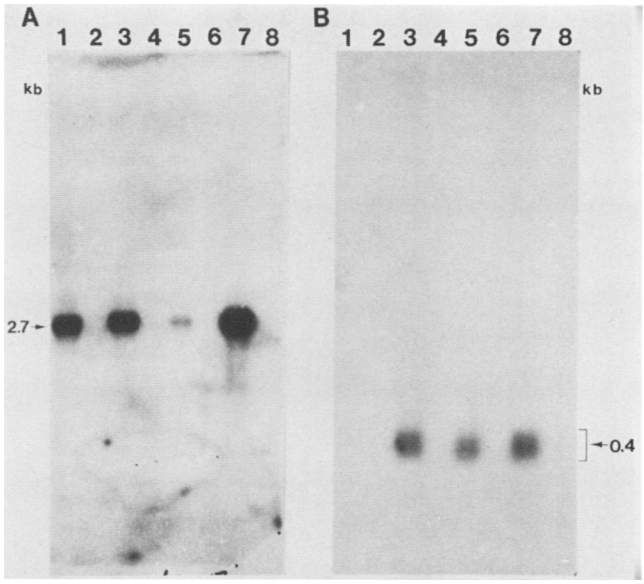

FIG. 4. Northern blot hybridizations of RNAs isolated from separated spermatogenic cells. RNAs were isolated from enriched populations of testicular cells in different stages of spermatogenesis, separated by sedimentation at unit gravity as described by Wolgemuth et al. (44), and analyzed by Northern blot hybridization as described in the legend to Fig. 2. The blots were hybridized with ^{32}P-labeled pMHS213 insert (panel A) or pMET1, a plasmid containing two copies of mouse metallothionein-1 cDNA (15) (panel B). Lanes: 1 and 2, 1.3 and 25 μg of poly(A)$^+$ and flowthrough RNAs from residual bodies and cytoplasmic fragments; 3 and 4, 4 and 30 μg of poly(A)$^+$ and flowthrough RNAs from early spermatids; 5 and 6, 4 and 30 μg of poly(A)$^+$ and flowthrough RNAs from pachytene spermatocytes; 7 and 8, 8 and 30 μg of poly(A)$^+$ and flowthrough RNAs from mature testes. Exposure time, 3 days for panel A and 7 days for panel B.

The 2.7-kb T-HSP70 transcript was abundant in RNA from early spermatids (Fig. 4A, lane 3) and the mixture of residual bodies and cytoplasmic fragments (lane 1). A very low amount of the 2.7-kb transcript was seen in the meiotic prophase spermatocyte fraction (lane 5), possibly reflecting either a low level of transcription at this stage or contamination of this fraction with multinucleated early spermatids (43). Neither of the typical somatic transcripts were detected in enriched germ cell populations.

The 2.7-kb T-HSP70 transcript was clearly more abundant in early spermatids than in any other enriched cell population (lane 3). The transcript was readily detected in the anucleate cytoplasmic fragments of elongating spermatids and the residual bodies (lane 1), although this sample contained approximately one-third the amount of RNA in the other samples. This indicated that the 2.7-kb T-HSP70 transcripts are stable throughout spermiogenesis. The same blot was rehybridized with a probe for mouse metallothionein-1 as a control for RNA integrity of the meiotic prophase fraction (Z. F. Zakeri, R. D. Palmiter, and D. J. Wolgemuth, unpublished data). Metallothionein-1 transcripts were easily detected in the meiotic spermatocyte (Fig. 4B, lane 5) and early spermatid (lane 3) fractions but not in the residual-body and cytoplasmic-fragment fraction (lane 1).

The 2.7-kb T-HSP70 is found in testes of other animals. RNAs were isolated from testes of other species to determine whether expression of the *hsp70* gene family in the male germ line is a common feature of mammalian develop-

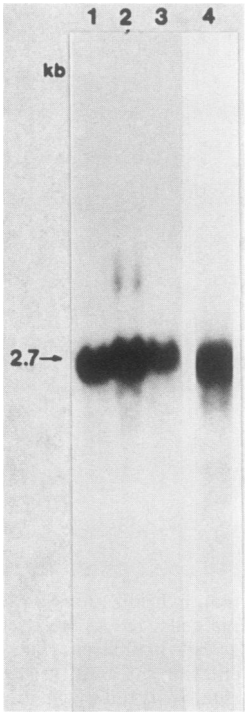

FIG. 5. *hsp70* transcripts in testes of other species. Testis RNAs were isolated from mature Swiss Webster mice (lane 1) and F_1 offspring of C57BL/6J × DBA matings (lane 2). Lane 3 contained RNA isolated from normal testicular tissue obtained as a discarded pathology specimen from an adult human male. Lane 4 contained RNA isolated from adult male Sprague-Dawley rat testes [10 μg of poly(A)$^+$ RNA]. Lanes 1 to 3 contained 20 μg of total RNA. Exposure time, 1 day.

ment. Abundant transcripts of ~2.7 kb were seen in RNAs isolated from testes of two different strains of mice (Fig. 5, lanes 1 and 2), from human testis (lane 3), and from rat testis (lane 4).

DISCUSSION

We have shown that a member of the *hsp70* gene family is expressed at high levels in mammalian testis. The transcript produced is unique in size and different from the two transcripts of 3.5 and 2.4 kb present at low levels in somatic tissues and from the major heat shock-inducible transcripts produced in stressed mouse L cells. This 2.7-kb transcript is present in testis in the absence of exogenous stress. Testis is a complex tissue, with a variety of somatic and germinal cell types. The transcript could either be produced constitutively at low to moderate levels in all cell types in testis or, alternatively, be expressed at higher levels in a subset of testicular cells. Three series of experiments allowed us to distinguish between these possibilities.

Our studies on a sterile strain of mice revealed that the 2.7-kb testis-specific transcript was lacking in RNA from testes devoid of germ cells. The two somatic transcripts 3.4 and 2.5 kb long were present at levels comparable to those seen in typical somatic tissues. Expression of this unique transcript within testis is thus restricted to germ cells.

The 2.7-kb testicular transcript was also lacking in testes from immature animals at day 7 to 8 of neonatal development. Although testes at this stage contained the full complement of somatic cells, as well as germ cells in the mitotic stem cell stages of spermatogenesis, they did not contain germ cells which had entered meiosis. This suggests that the transcript is restricted to germ cells which have entered meiosis. This developmental specificity of expression is further underscored by the fact that the 2.7-kb transcript was also undetectable in testes from animals at day 17 of postnatal development. Testes at this age contain germ cells which have entered meiosis and are just completing the first and second meiotic reduction divisions. From this point onward, the number of cells in the haploid stage of spermatogenesis increases. Concomitantly, the 2.7-kb transcript appears in adult testes. The presence of the 2.7-kb transcript is thus coincident with the entry of the cells into the haploid stage.

Further support for this conclusion was obtained by examining the HSP transcripts in enriched populations of spermatogenic cells. The 2.7-kb transcript was present in populations of early (round) spermatids, in the cytoplasmic fragments of elongating spermatids, and in residual bodies. Little or no transcript was detected in RNA isolated from enriched populations of meiotic prophase spermatocytes. Transcription ceases several days before sloughing of the cytoplasm to form the residual body. The level of the 2.7-kb transcript in the cytoplasmic fragment and residual body fractions suggests that the transcript is very stable. Experiments are in progress to determine whether the *hsp70* family member protein is translated early in the process of spermiogenesis or at the terminal stages of testicular sperm development.

The observation of a unique transcript at the haploid stage of spermatogenesis places the HSP gene family into the category of genes exhibiting haploid-stage-specific expression. These genes include the testis-specific variants of the alpha-tubulin and actin genes (12, 40), the nuclear protein-encoding genes protamine 1 and 2 (20), and the proto-oncogene c-*abl* (33), as well as an unidentified haploid-stage-specific cDNA (13).

It is interesting to compare the pattern of expression during spermatogenesis of this member of the mammalian *hsp70* family with the pattern of the *Drosophila hsp70* gene (8). The *hsp70* promoter is inducible by heat shock in spermatogonial stem cells of *Drosophila* spp. but not in cells that have entered meiosis. No developmental induction of the *hsp70* promoter during *Drosophila* spermatogenesis has been reported. In contrast, our observations demonstrate that a member of the *hsp70* family is specifically and abundantly expressed in a precise developmental pattern during mammalian spermatogenesis. Its pattern of expression in the male germ line suggests a role in postmeiotic (haploid) phases of gametogenesis. The generality of this observation within mammalian systems is demonstrated by the presence of a transcript from the *hsp70* family in testes from different strains of mice, as well as in rat and human testes. In addition, recent studies from our lab (unpublished data) and others (R. L. Allen and E. M. Eddy, Abstr. Annu. Meet. Am. Soc. Cell Biol. 1986, 103, p. 81a), using antibodies to *hsp70* and two-dimensional gel electrophoresis, demonstrate the presence of ~70-kd proteins in testes.

This cellular and developmental specificity of expression should help in elucidating the function of this member of the *hsp70* gene family, particularly its role in germ cell differentiation. Our results and observations of others, particularly in *Drosophila* spp., suggest that heat shock proteins have basic functions that are part of the normal development of germ cells.

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