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Cloning of a *hsp70*-related gene expressed in mouse spermatids

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A cDNA library of spermatids was screened by a differential hybridization in order to isolate genes expressed in haploid cells of the mouse male germ line. A clone was found that encoded a protein related to the heat shock protein 70. A genomic DNA clone comparable to this cDNA clone was also isolated from a mouse genomic library. This gene had only one continuous open reading frame capable of coding a 630 amino-acid protein. There was an excellent match of the sequence with the heat shock protein 70 family but a difference from any previous 70 kilodalton heat shock protein. A 2.7kb transcript derived from this gene was expressed in spermatids but not in other testicular germ cells and somatic tissues. We have referred to this gene as *hsc70t*. © 1990 Academic Press, Inc.

Spermatogenesis is a complex series of developmental events in which mitotic proliferation of spermatogonia is followed by meiosis of spermatocytes and differentiation of haploid spermatids to mature spermatozoa. The distinct morphological feature and specialized function of the spermatozoa are obtained during final steps of spermiogenesis and supported by a large number of proteins that are not found in any other cell type (1). Spermiogenesis may require a fundamental reprogramming of gene expression. A prerequisite to the genetic analysis of the construction of the complex structure in spermatozoa, the genes involved in the spermiogenesis should be identified and mechanisms of their expression should be ensured.

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Abbreviations: HSP70, heat shock protein 70 kilodalton; *hsc*, heat shock protein cognate gene.

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Recombinant DNA techniques have been used for molecular cloning of genes expressed during mammalian spermatogenesis (2, 3, 4). Here, we have isolated a mouse cDNA clone homologous to *hsp70* genes which is expressed specifically in spermatids. A genomic DNA clone has been also isolated and its relationship to other members of the *hsp70* gene family was examined at the level of DNA sequence analysis.

Materials and Methods

Preparation of testicular germ cells: Sexually mature male mice were used for preparation of testicular germ cells by the two step method with collagenase and trypsin, as described previously (1). Centrifugal elutriation was performed on the testicular germ cells using a Beckman JE-6 elutriator roter (5).
Cell culture and heat shock conditions: Mouse L*tk*$^-$ cells were grown in Eagle minimal essential medium with 10% fetal bovine serum at 37°C with 5% CO_2. For the heat shock treatment, the cells were kept at 44°C for 90 min and allowed to recover for 2.5hr at 37°C.
Extraction of RNA: RNA was isolated from cells and tissues by the guanidium thiocyanate/ CsCl method (6). Poly(A)$^+$ RNA was selected through two cycles of oligo(dT) cellulose chromatography (6).
Construction of a spermatid cDNA library and a mouse genomic library: Poly (A)$^+$ RNA of early spermatids isolated by elutriation was used for construction of a cDNA library with lambda phage λgt10 as a vector (6). The mouse genomic library (a gift from Dr. A. Matsushiro) was constructed in a lambda phage EMBL4 vector by partial digestion of 129/SvJ mouse DNA with *Eco*RI.
DNA sequence analysis: DNA fragments were subcloned into a pUC19 and nucleotide sequences were determined by Sanger's method (7).
Northern and RNA slot-blot analysis: For Northern analyses poly(A)$^+$ RNA was subjected to electrophoresis in 1% agarose-formaldehyde gel (6) and transferred to Biodine A nylone membrane (Nihon Pall, Japan). For the RNA slot blot analysis total RNA was transferred to nylon membrane using a microsample filtration manifold device (Schleicher and Schuell). Filters were hybridized with appropriate DNA probes labelled with ^{32}P by Amersham multiprime DNA labelling system.

Results and Discussion

The spermatid cDNA library was screened by a differential hybridization method using ^{32}P-labelled cDNA probes to poly(A)$^+$RNA prepared from either spermatids or immature (17-day) decapsulated testes, which had no spermatids. Several clones hybridized with the spermatid probe were picked and analyzed with Northern blots of RNA isolated from spermatocytes and spermatids. One of these clones, HS2, gave clear indications of being expressed abundantly in haploid cells (see below).

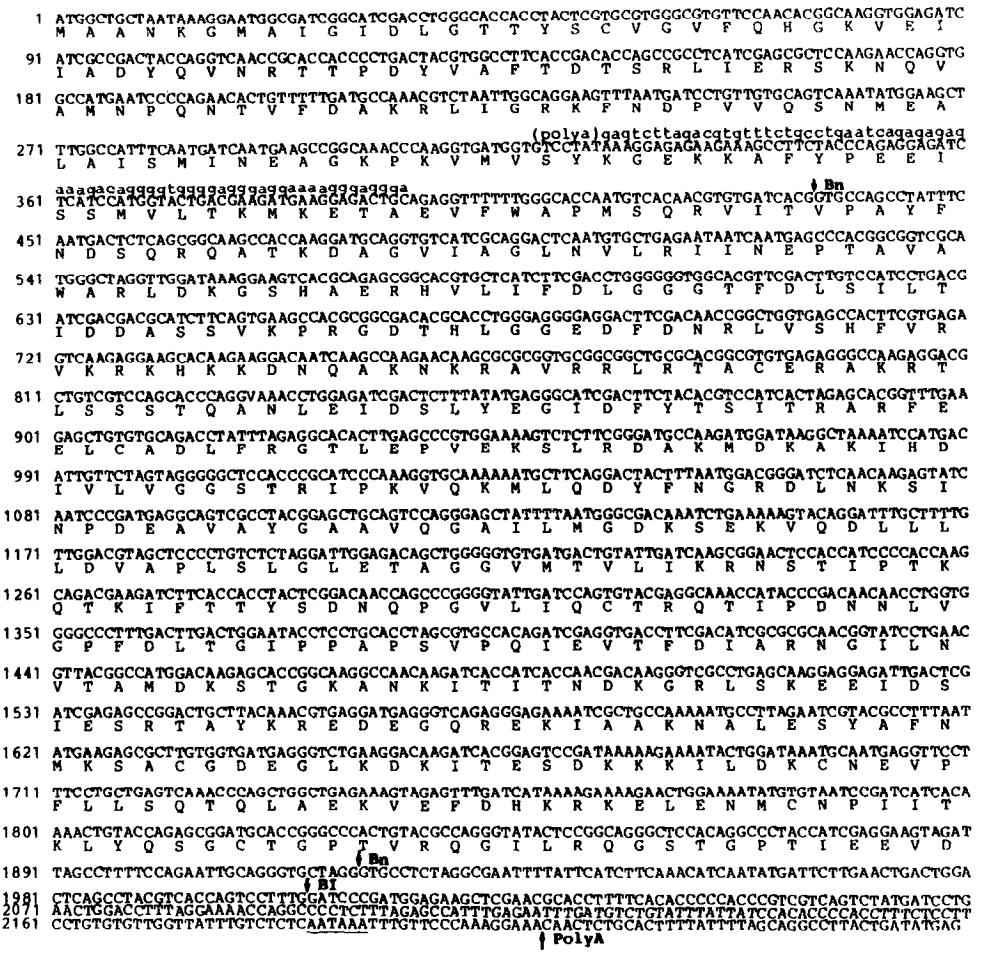

Fig. 1. Nucleotide sequence of the mouse *hsc70t* gene and the deduced amino acid sequence, as determined from the genomic GE6 and the HS2 cDNA clones. A nucleotide sequence shown in a small letter was derived from the 5' end of the HS2 cDNA clone. Polyadenylation signal is underlined. PolyA; addition site of a polyadenylation tract, Bn; *Ban*I site, BI; *Bam*HI site.

DNA sequencing analyses and computer searching the protein sequence database of the NBRF revealed that the HS2 clone contained a 1.9 kb insert with an amino acid coding region homologous to the carboxyl terminal residues of the *Xenopus* HSP70 and a 3' untranslated region including a polyadenylation signal (Fig. 1).

We screened a genomic DNA library (4.5 x 10^5 clones) using the 3' untranslated region (*Bam*HI-*Eco*RI 200bp) of the HS2 cDNA clone as a probe and isolated 2 positive clones. The restriction mapping showed that they shared common restriction sites. The 3' untranslated probe

of the HS2 cDNA detected a strong band corresponding to the *Eco*RI 6kb fragment length with mouse genomic DNA (not shown). Partial DNA sequencing of the *Eco*RI 6kb fragment (GE6) of the genomic clone has provided a perfect match for stretches of the HS2 cDNA clone except for an about 70bp sequence in 5' region of the HS2 cDNA nucleotides (Fig. 1). This mismatched region of the HS2 cDNA might be caused by artifact of cDNA cloning. This gene has only one continuous open reading frame of 1,893 nucleotides starting at ATG and extending to the termination codon TAG, which is capable of coding 630 amino-acid protein with a predicted molecular weight of 69,645. The amino acid sequence deduced from this open reading frame had a very good fit with the HSP70 family but differed from any previously sequenced mouse HSP70 family proteins (Table 1). We have referred to this gene as *hsc70t*.

To examine whether there is a tissue-specific expression of the *hsc70t* gene, total cellular RNA isolated from various tissues were analyzed by the slot-blot hybridization with the 3' untranslated region unique probe. Figure 2A shows *hsc70t* mRNA expressed specifically in the testis. Recent reports have shown that testes of adult rat and mouse contain abundant levels of the transcript related to the *hsp70* gene (9, 10, 11, 12). Expression of the *hsc70t* mRNA was also analyzed by Northern blot hybridization (Fig. 2B). RNA was isolated from mouse L*tk*$^-$ cells, heat-shocked L*tk*$^-$ cells, and enriched populations of pachytene spermatocytes and early spermatids.

TABLE 1. Similarity of the deduced amino acid sequence among mouse *hsp70* genes

hsp70 gene	% similarity of *hsc70t*	(reference)
hsc70 (*hsc71*)	72.7	(8)
hsp68 (MHS243)*	72.9	(13)
HSP70.2	71.1	(11)

* A cloned gene encodes only 418 carboxyl terminal amino acids.

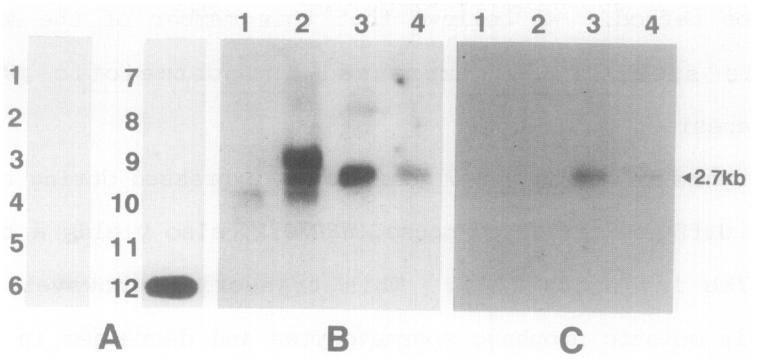

Fig. 2. *hsp70*-related gene transcripts in normal mouse tissues, enriched populations of spermatogenic cells and mouse L cells. A: Total RNA (10µg each) were blotted on the filter and hybridized to a probe derived from the 3' untranslated region of the *hsc70t*. 1; heart, 2; brain, 3; liver, 4; kidney, 5; pancreas, 6; spleen, 7; lung, 8; stomach, 9; intestine, 10; ovary, 11; epididymis, 12; testis. B: Poly(A)$^+$RNA (1µg each) were electrophoresed in a 1.0% denatured agarose gel and processed for Northern blot hybridization analysis with ^{32}P-labelled probe derived from the coding region of the *hsc70t*. lane 1; L*tk*$^-$ cells, lane 2; heat shocked L*tk*$^-$ cells, lane 3; spermatids enriched by elutriation, lane 4; spermatocytes enriched by elutriation. C: The Northern filter same to B was hybridized to the probe derived from the 3' untranslated region of the *hsc70t*.

A probe (*Ban*I 1500bp) from the codig region of HS2 detected two major heat-inducible transcripts and a less abundant transcript, which was present in both induced and noninduced L*tk*$^-$ cells. In male germ cells, transcript of about 2.7kb was observed. This transcript was more abundant in the early spermatid population than in the pachytene spermatocyte population. A similar pattern of expression has been observed when RNAs from the same sources were hybridized with pMHS213, a cDNA clone isolated from a heat-shocked L-cell (9, 13). When the same filter was hybridized with the 3' untranslated region fragment (*Bam*HI-*Eco*RI) of HS2 as a probe, transcripts were not recognized in the RNA from the L*tk*$^-$ cells. However, abundant expression of the transcript of about 2.7kb was observed in the early spermatids (Fig. 2C). In the spermatocyte population, this unique probe detected a very faint 2.7kb transcript. The amount of transcript seen in the spermatocytes could be derived from the multi-nucleated spermatids which contaminate the fraction of spermatocytes enriched by the

elutriation method. We believe that this member of the *hsp70* gene family is specifically expressed in postmeiotic phases of spermatogenesis.

Another member of the *hsp70* gene family expressed during testicular germ cell differentiation of mouse, *HSP70.2*, also yields a transcript about 2.7kb in length (11). This transcript, however, is most abundant in meiotic prophase spermatocytes and decreases in abundance in postmeiotic stages of spermatogenesis. A novel mouse HSP70-like protein, P70, is shown to be primarily synthesized in pachytene spermatocytes and slightly in early spermatids (14, 15). The P70 protein may be encoded by the *HSP70.2* gene.

Thus, expression of two members of the *hsp70* gene family is sequentially regulated during the mouse male germ cell differentiation. Further studies will be needed to determine the cellular localization of the product during spermiogenesis. The DNA clone for the *hsc70t* gene will facilitate analyses of mechanisms of haploid gene transcription in the male germ cell lineage.

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