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Developmentally Regulated Expression of APG-1, a Member of Heat Shock Protein 110 Family in Murine Male Germ Cells

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***Apg-1* encodes a heat shock protein belonging to the heat shock protein 110 family, and is inducible by a 32°C to 39°C heat shock. Northern blot analysis of the testis from immature and adult mice, and of the purified germ cells revealed the quantitative change of the *apg-1* transcripts during germ cell development. By in situ hybridization histochemistry the expressions of the *apg-1* transcripts were detected in germ cells at specific stages of development including spermatocytes and spermatids. Although heat-induction of the *apg-1* transcripts was observed in *W/W*^{v} mutant testis lacking germ cells, it was not detected in wild-type testis nor in the purified germ cells. Thus, the *apg-1* expression is not heat-regulated but developmentally regulated in germ cells, suggesting that APG-1 plays a role in normal development of germ cells.**

Prokaryotic and eukaryotic organisms respond to elevated temperatures and other cytotoxic conditions by synthesizing a distinct set of proteins, termed heat shock proteins (HSPs) (1). HSPs are assumed to protect cells from the harmful effects of stress conditions. HSPs play important roles even in non-stressed cells, and members of HSPs are now well established as molecular chaperons (2). The major classes of mammalian HSPs are HSP90s (83-99 kDa), HSP70s (68-80 kDa), HSP60s and the smaller HSPs (25-28 kDa) (1). Recently, a novel cDNA encoding 110 kDa heat shock protein (*hsp110*) was cloned from chinese hamster and mouse (3)(4). Subsequently, from a mouse testis cDNA library, we have isolated novel HSP-encoding cDNAs, *apg-1* and *apg-2*, which will constitute a distinct hsp family together with *hsp110* (5)(6). Independently, apg-1 was identified as hyperosmolarity-induced gene *Osp94* by Kojima et al. (7).

Spermatogenesis begins at puberty and consists of three steps; mitotic proliferation of spermatogonia, meiosis at spermatocyte stage and distinct structural changes at spermatid stage (8). Several HSPs have been supposed to be involved in normal germ cell development (9-12), while the significance of HSPs in the protection of germ cells from thermal stress is uncertain (13)(14). The testis temperature is maintained to be lower than the body cavity temperature (15). The artificial replacement of the mouse testis in the body cavity, or treatment of the lower body at 42°C selectively damage the germ cells (16)(17). We previously reported that the optimal heat conditions for the induction of *apg-1* transcripts are different from those of *hsp70* transcripts (5). The *apg-1* transcripts are induced in cultured somatic cells by the temperature shift from 32°C to 39°C but not by the traditional 37°C to 42°C shift. In this study, we examined the expression of the *apg-1* transcripts in testicular somatic and germ cells during spermatogenesis and in response to thermal stress.

MATERIALS AND METHODS

Mice. Sexually immature (3-day and 7-day old) and Sexually mature (4-month old) ddy/std mice, and WBB6F1-*W/W*^{v} mutant mice were purchased from Japan SLC Company (Hamamatsu, Japan).

Fractionation of testicular germ cells. Purified germ cell populations were obtained from the testes of 4-month old ddy/std mice using centrifugal elutriation and Percoll density gradient separation methods, as described previously (18).

Heat treatment of testis tissues and purified germ cells. Testes from sexually mature ddy/std mice and *W/W*^{v} mutant mice, and the purified germ cell fraction containing spermatocytes and spermatids were incubated in the Dulbecco's modified Eagles medium supplemented with 1% non-essential amino acids and 10% fetal calf at 32°C, 37°C or 39°C for 2 h in a CO2 incubator.

RNA extraction and Northern blot hybridization. RNA was extracted as described (18). Aliquots of RNA samples were separated

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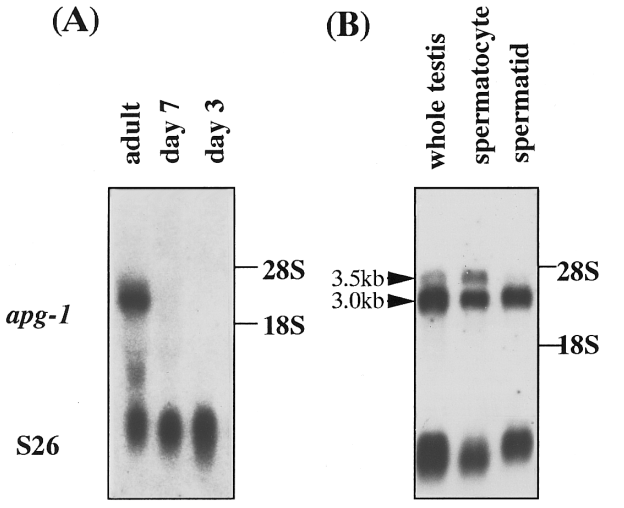

FIG. 1. (A) Expression of *apg-1* transcript in the testes of 4-month old (adult), 7-day old (day 7) and 3-days old (day 3) mice. Each lane contained 20 μg of total RNA. (B) Expression of the *apg-1* transcript in 4-month old mouse testes (whole testis), pachytene spermatocytes and round spermatids. Each lane contained 4 μg of poly(A)+RNA. The probe used was the 1873-bp *Eco*RI-*Dra*II fragment from *apg-1* cDNA (5). The filters were subsequently rehybridized with a probe for the S26 ribosomal protein to correct for the amount of RNA loaded.

on 1.0% agarose/formaldehyde gels by electrophoresis and were blotted onto nylon filters (Hybond-N+; Amersham, Buckinghamshire, UK). The filters were hybridized as described previously (18).

In situ hybridization histochemistry. A 1873-bp *Eco*RI-*Dra*II fragment of *apg-1* cDNA (5) was cloned into the vector pBluescript SK(−) (Stratagene, La Jolla, CA) and was used as a probe. Fixation and embedding of tissues and in situ hybridization were done as previously described (18).

RESULTS

Expression of apg-1 Transcripts in Male Germ Cells

To elucidate which cell types in the testis express *apg-1*, we performed Northern blot analysis using RNAs extracted from testes of immature mice (3-day, 7-day) and adult mice (4 month old), and purified male germ cells of adult mouse. Testicular tissues from 3-day and 7-day old mice contain only gonocytes and spermatogonia, respectively, as a germ cell compartment together with somatic cells. As shown in Figure 1A, the expression of *apg-1* was detected in the adult testis but not in the immature testis, suggesting that the *apg-1* transcripts are abundantly expressed in differentiated germ cells of the adult testis. Figure 1B shows that the *apg-1* transcripts were expressed in purified pachytene spermatocytes and round spermatids. The 3.5 kb *apg-1* transcripts were detected in spermatocyte but not in spermatids. These results demonstrated that the *apg-1* transcripts were expressed in germ cells and the amounts of the *apg-1* transcripts changed depending on the stage of germ cell development.

In situ Determination of Cells Expressing apg-1 Transcripts in the Testis

To further determine the localization of the *apg-1* transcripts in the testes, we carried out *in situ* hybridization histochemistry. Testicular tissues from 3-day, 7-day and 4-month old wild-type mice were sectioned and used for this analysis. Figure 2 shows the results using *apg-1* as probes. No significant signals were observed in the testes from 3-day and 7-day old mice using an antisense cRNA probe as compared with those using a sense cRNA probe (Figs. 2 a–d). In the testes of wild- type adult mice, signals were detected in the pachytene spermatocytes and round spermatids but not in the spermatogonia or somatic cells (Fig. 2e). No significant signals were detected when the sense *apg-1* cRNA was used as a probe (Fig. 2f). These data were in agreement with the results obtained from the Northern blot analysis.

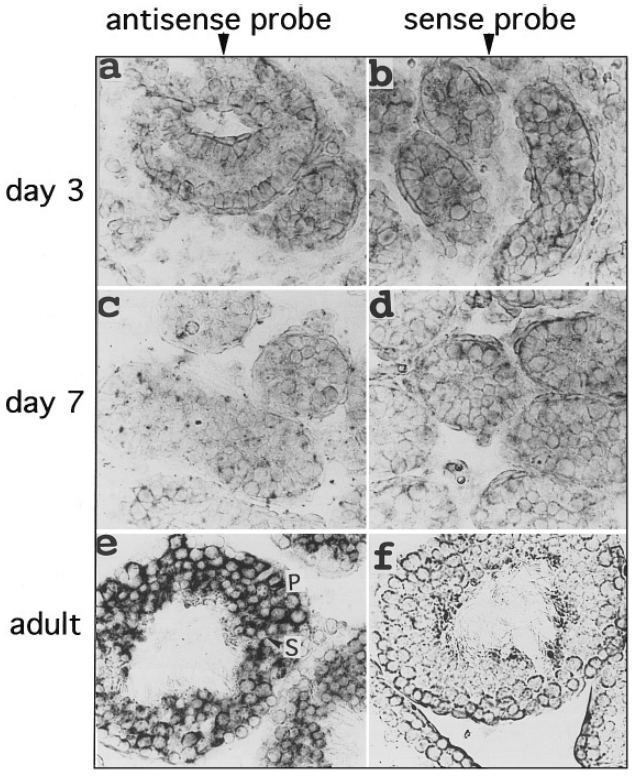

FIG. 2. *In situ* hybridization histochemistry of the *apg-1* transcript in the mouse testes. The 1873-bp *Eco*RI-*Dra*II fragment from *apg-1* cDNA (5) was used to generate the cRNA probes. (a) and (b), sections from 3-day old ddy/std mouse testes; (c) and (d), sections from 7-day old ddy/std mouse testes; (e) and (f), sections from 4-month old ddy/std mouse testes. (a), (c) and (e): hybridization with the antisense cRNA strand from *apg-1*; (b), (d) and (f): hybridization with the sense cRNA strand from *apg-1*. S, round spermatid; P, pachytene spermatocyte.

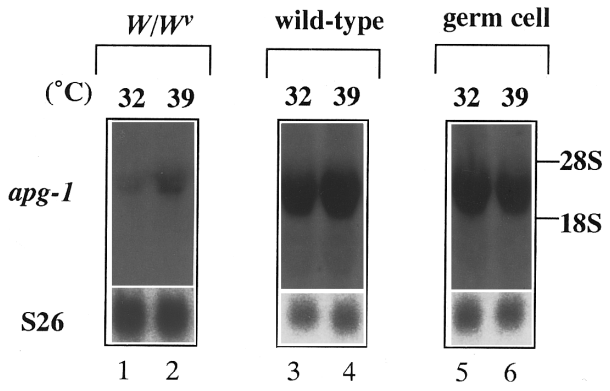

FIG. 3. Lack of heat-induction of *apg-1* transcripts in germ cells. Each lane contained 40 μg of total RNA extracted from testes of W/W^v mutant mice (lanes 1 and 2), or 20μg of total RNA extracted from testes of wild-type mice (lanes 3 and 4) or from purified germ cell fraction containing round spermatids and pachytene spermatocytes (lanes 5 and 6). RNAs were extracted from indicated tissues or cells after incubation for 2h at 32°C (lanes 1, 3 and 5) or 39°C (lanes 2, 4 and 6). The filters were hybridized with the *apg-1* cDNA probe, and then rehybridized with a probe for the S26 ribosomal protein.

Lack of Heat-Induction of apg-1 Transcripts in Wild-Type Testis and Purified Germ Cells

We examined the induction of the *apg-1* transcripts by temperature shift in the wild-type and W/W^v mutant mouse testes and purified germ cells in vitro. The relative amounts of RNA contributed by somatic cells in the wild-type testis are much smaller than those by germ cells, while the W/W^v mutant mice lack germ cells and the testicular RNAs are derived only from somatic compartment (18). Northern blot analysis of the testis from W/W^v mutant mice demonstrated that the amounts of both 3 kb- and 3.5 kb-*apg-1* transcripts increased by the temperature shift from 32°C to 39°C (Fig. 3, lanes 1 and 2). In contrast, no change in the amount of *apg-1* transcripts was observed in the wild-type testis by the up-shift from 32°C to 37°C or 39°C (Fig. 3, lanes 3 and 4; data for 37°C not shown). The *apg-1* transcripts were not heat-induced in the purified germ cells including both pachytene spermatocytes and round spermatids, either (Fig. 3, lanes 5 and 6). No histological changes were detected in the testes after incubation at 39°C for 2 h (data not shown). These results suggest that *apg-1* is induced in testicular somatic cells but not in germinal compartment of the testis.

DISCUSSION

To date, several HSPs have been found to be constitutively expressed in male germ cells at specific stages of development. Two *HSP70*-related genes, *hsp70.2* and *hsc70t* are expressed in spermatocytes and spermatids, respectively (9)(10). HSP90 and HSP60 are expressed in spermatogonia and spermatocytes (11)(12). A recent study using gene targeting technique demonstrated that male mice devoid of HSP70.2 lack spermatids and are infertile (19). These findings suggest that HSPs play a role in normal germ cell development. We demonstrated that the *apg-1* transcripts were expressed in germ cells undergoing meiosis, including pachytene spermatocytes and round spermatids, but not in spermatogonia (Figs. 2 and 3). This quantitative change in the expression of *apg-1* during germ cell development suggests that *apg-1* also plays a role in spermatogenesis. Pachytene spermatocytes expressed two types of *apg-1* transcripts, 3.5kb and 3.0kb in size, while spermatids expressed only the latter. (Fig. 2b). The two types of transcripts probably contain poly(A) tails of different length (7). Alternative polyadenylation resulted from the removal of an A/U-rich motif has been shown to stabilize the shortened transcripts (20). Thus, the 3.0kb-*apg-1* transcript may be essential for the translational delay found in post-meiotic male germ cells.

The fact that elevation of scrotal temperature selectively damages germinal compartment of the testis suggests a differential heat-sensitivity between somatic cells and germ cells of the testis (21). Even among germ cell populations, a differential heat-sensitivity has been observed; spermatogonia and spermatozoa are the most resistant, whereas spermatocytes and spermatids are the most vulnerable (21). To elucidate this mechanism for the differential heat-sensitivity, several studies have examined the heat-inducibility of *hsps* in germ cells with conflicting results (13)(14). Recently, the temperature threshold for induction of HSP70 has been demonstrated to be lower in pachytene spermatocyte (38°C) than in somatic cell type of the testis (42°C)(22). Our previous study, however, demonstrated that *hsp70*, *hsp110* and *apg-1* transcripts are inducible by the temperature shift from 32°C to 39°C even in somatic cells (5). We showed in the present study that the induction of the *apg-1* transcripts were detectable in the testes of W/W^v mutant mice but not those of wild-type mice or the purified germ cells after the temperature shift from 32°C to 39°C. These results suggested that the *apg-1* expression is differently regulated in somatic and germ cells of the testis. Since the apg-1 transcripts were abundantly expressed in the heat-vulnerable germ cell populations irrespective of heat stress, APG-1 protein may not affect the heat-sensitivity of male germ cells.

Consensus cis-acting element, heat shock element (HSE) is required for proper induction of the *hsp* transcripts by stress and/or development (23). Two transcription factors, heat shock factor 1 (HSF1) and HSF2 have been shown to interact with HSE in mammalian cells (24). Since the HSEs are also present in the 5′ flanking region of *apg-1* gene (5), the interactions between HSEs and HSFs should further be explored to clarify the mechanisms regulating the differential ex-

pression of *apg-1* in somatic cells and germ cells of the testis.

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