

## Expression of Small Stress Protein Hsp20 Gene in the Maturing Rat Testis

Yoshiaki YAMANO<sup>1)</sup>, Kenji OHYAMA<sup>2)</sup>, Masanori OHTA<sup>2)</sup>, Juri NAKAMURA<sup>3)</sup> and Isao MORISHIMA<sup>3)</sup>

<sup>1)</sup>Laboratory of Veterinary Biochemistry, Faculty of Agriculture, Tottori University, Tottori 680-8553, <sup>2)</sup>Interdisciplinary Graduate School of Medical and Engineering Sciences, University of Yamanashi, Yamanashi 409-3898 and <sup>3)</sup>Department of Biochemistry and Biotechnology, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

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**ABSTRACT.** In this study, we cloned a cDNA that encodes a small heat shock protein, Hsp20 ( $\alpha$ B crystallin-related protein), from a maturing rat testis by means of differential display. The full-length cDNA sequence was completely identical to that registered in the DNA databank. The expression of Hsp20 gene was detected strongly in the heart and slightly in the testis of a 9-week-old rat. The expression of Hsp20 increased gradually from three weeks to 9 weeks, and the strongest expression was observed in the testis at week fifteen. The expression was localized in spermatocytes and round spermatids. The gene expression was not affected by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) when it was administered into male rats during the nursing period.

**KEY WORDS:** Hsp20, spermatogenesis, TCDD.

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Heat shock proteins (HSPs) are molecular chaperones that have many physiological roles for protecting cells from environmental hazards, such as heat and chemicals. These proteins are expressed in many organs. In the reproductive system, many genes are systematically expressed in an array, from diploid spermatogonia to haploid spermatids. Spermatogenesis is a complex process composed of mitosis, meiosis, and cellular differentiation. During the reproductive process, the testicular environment should be shielded from environmental pollutants or diverse stressors for cellular division and differentiation to proceed normally. Many kinds of HSPs are expressed in the testis, as well as the ovary [3]. The expression of Hsp70 and Hsp90 was up-regulated in spermatogenic cells exposed to elevated temperature [9, 18]. The temperature of the testes should be regulated lower than that of other organs in animals, and even a slight elevation of scrotal temperature is associated with male infertility inducing apoptosis of spermatogenic cells, such as in cryptorchid patients [9, 12, 16]. Critical regulation of the temperature during spermatogenesis is important, and during this period, many kinds of HSPs are expressed to establish a normal spermatogenic process. Among the HSP groups, there is a novel group of small heat shock proteins (sHSPs). These proteins have a conserved C-terminal  $\alpha$ -crystallin domain [1], and up to date, ten genes that encode sHSP have been characterized in the human genome [5]. Among them, Hsp27, HspB5 ( $\alpha$ B-crystallin), and Hsp20 (HspB6, p20) are expressed mainly in muscles, and they have important physiological roles in muscle maintenance [4, 17, 19]. Another sHSP protein called sperm outer dense fiber protein (ODFP), or HspB10, is expressed in human sperm cell tails, and may have cytoskeletal functions [2]. Among the sHSP family, Hsp20 has the unique characteristics of being phosphorylated, and composed of multimers that induce relaxation of vascular smooth muscle, and it has poor chaperone-like activity, compared to other members of the sHSP family [14, 20]. Interestingly, some

sHSP family member proteins are expressed ubiquitously, and some are testis specific [6]. Currently, the physiological importance of Hsp20 in the reproductive system when stressors are applied has yet to be clarified. In this report, we have characterized the expression profile of Hsp20 in the testis during the developmental stage, and determined the expression in testis tissue and in the TCDD administered rat testis.

Male Sprague-Dawley (SD) rats were housed in an air-conditioned animal room at an ambient temperature of 22°C, a relative humidity of 55  $\pm$  10%, and a 12-hr light/dark cycle. Animals received food and water *ad libitum*. All animal experiments were approved by the University of Yamanashi Animal Care and Use Committee. A cDNA fragment that was specifically expressed in the testis of a 7-week-old rat was compared with that of a 3-week-old rat based on the results of differential display (DD) [10], and was cloned using an anchored primer (T<sub>12</sub>AA) and a random primer (OP26-04: TTTTGCTCC). A detailed DNA analysis was performed on one clone that was expressed in the testis of the 7-week-old rat, and the sequence was completely matched with rat Hsp20 (HspB6) cDNA [4]. The coding region of this gene was synthesized according to the reported data (DNA database accession number D29960, NM\_138887) by PCR. Total RNA from the SD rat testis and from individual organs of a 9-week-old animal was prepared. Five  $\mu$ g of the individual RNA was electrophoresed in a gel containing formaldehyde and was transferred to a nylon membrane, Hybond N<sup>+</sup> (Amersham Biosciences Corp, NJ, U.S.A.). The cDNA that encodes Hsp20 was labeled with a random primer labeling system (Amersham Biosciences) using [ $\alpha$ -<sup>32</sup>P]dCTP and was used as a probe. Hybridization was performed, and the blot was rehybridized with rat  $\beta$ -actin cDNA as an internal standard as reported previously [22]. Testicular expression of Hsp20 was determined by *in situ* hybridization of a section of testis of an 8-week-old rat, following the same procedure described previ-

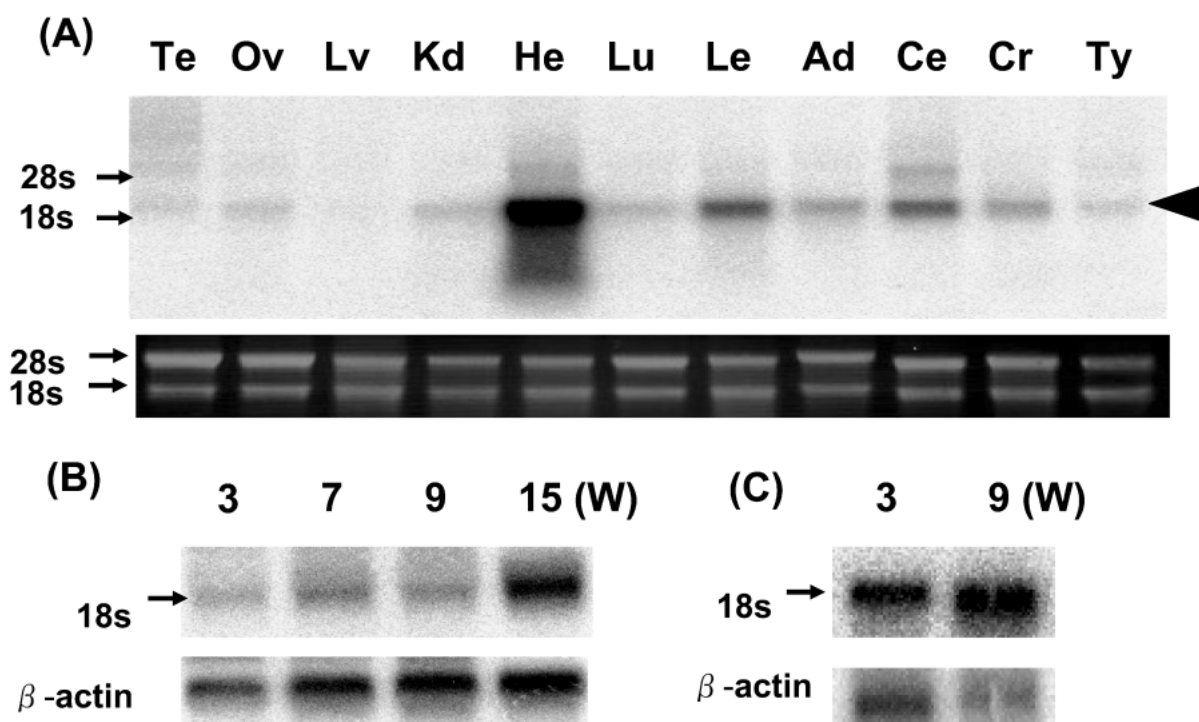


Fig. 1. (A) Northern blot analysis of the RNA from various rat organs (Te: testis, Ov: ovary, Lv: liver, Kd: kidney, He: heart, Lu: lung, Le: lens, Ad: adrenal gland, Ce: cerebellum, Cr: cerebrum, and Ty: thymus), using Hsp20 cDNA as a probe. RNAs (5  $\mu$ g for each lane) from a 9-week-old rat were electrophoresed. Ribosomal RNA positions are shown with arrows on the left side as 28S (28S rRNA) and 18S (18S rRNA), and the position of the Hsp20 mRNA signal is indicated as an arrowhead on the right side. To confirm the integrity and quantity of the RNA, the denaturing gel electrophoresis pattern of each RNA sample is indicated at the bottom. (B) Northern blot analysis of the RNA at various postnatal stages for the rat testis using Hsp20 cDNA as a probe. RNAs (5  $\mu$ g for each lane) from the testes of 3-, 7-, 9- and 15-week (W) old rats were electrophoresed. To confirm the integrity and quantity of the RNA, the blot was rehybridized with rat  $\beta$ -actin cDNA, as indicated at the bottom. (C) Northern blot analysis of the RNA from the heart of three and nine-week old rats, using Hsp20 cDNA as a probe. RNAs (5  $\mu$ g for each lane) were electrophoresed. To confirm the integrity and quantity of the RNA, the blot was rehybridized with rat  $\beta$ -actin cDNA, as indicated at the bottom.

ously [22]. TCDD (AccuStandard, Inc., CT, U.S.A.) was dissolved in toluene as a stock solution and was diluted in corn oil in an appropriate concentration before use. Maternal rats were subcutaneously injected with a 300 ng/kg or 1,000 ng/kg dose of TCDD each week, three times after birth, and then, the pups were injected with the same dose of TCDD each week from ages three to eight weeks. At week nine, the testes were removed, the Hsp20 gene expression was determined by Northern blotting using the same procedure as described previously [22], and the radioactivity of each signal was determined by a FLA-5000 Fuji fluoromage analyzer (FUJIFILM Co., Ltd., Tokyo, Japan). The intensity of the signals was standardized by using the rat  $\beta$ -actin signal intensity. Results are presented as means  $\pm$  SE. Statistical significance was determined by Student's *t*-test.

The expression of Hsp20 gene was strongest in the heart, moderate in the lens and brain, and weak in the ovary and testis (Fig. 1-A). Some of sHSP genes are expressed in muscle-related tissues, and the strong expression of Hsp20 in the heart muscle was reasonable [4, 6, 17, 19]. In this experiment, we cloned the Hsp20 gene by means of DD, and it is consistent that the gene expression was higher during the

stage of puberty, seven-week-old rats, compared with that of three-week-old rats during the nursing period. The Hsp20 expression in the testis increased gradually from the postnatal date, and the maximum expression was observed at week 15 (Fig. 1-B). In the case of the gene expression in the heart, the level was higher at week nine compared with that at week three (Fig. 1-C). The protein concentration in the hindlimb muscle of rats was increased during the developmental stage, as reported by Inaguma *et al.*, although they did not show data concerning gene expression in the heart and testis [4]. As there are few reports concerning the physiological importance of sHSPs in the testis, we could not elucidate why the gene expression was up-regulated so much at week 15. To understand this phenomenon, the locus of the gene expression in the testis was determined by *in situ* hybridization. The expression in the testis was clearly visible in round spermatids and spermatocytes, even though the signal intensity was weak in the Northern blotting (Fig. 2). One small stress protein was expressed in the sperm cell tails, named as ODFP or HspB10, and this protein was related to the cytoskeleton [2]. Hsp20 may have a similar physiological function as ODFP.

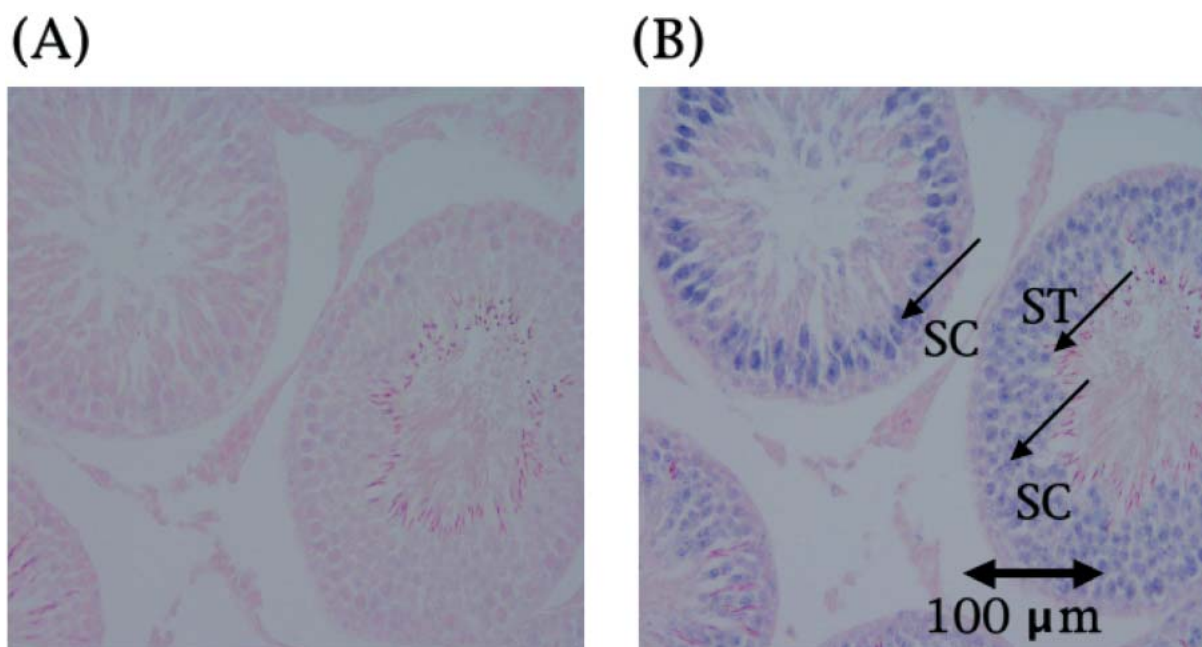


Fig. 2. Cellular localization of the Hsp20 transcript in the eight-week-old rat testis. *In situ* hybridization of a DIG-labeled sense (A) and antisense (B) rat Hsp20 cRNA probe to the 5- $\mu$ m sections of rat testis. Signals (purple color) in the spermatocytes (SC) and round spermatid (ST) are indicated by arrows. Cells are counterstained with nuclear fast red (red color). The 100  $\mu$ m size is shown in the figure by a double arrow.

We cloned spermatogenesis related genes that are expressed in maturing rat testes, and are interested in the effects of endocrine disruptors on the gene expression. We have reported two genes, SRF-1 [21] and SRF-2 [22], that are expressed in maturing rat testes. Both of these genes are expressed in spermatocytes, based on data from *in situ* hybridization. The deduced SRF-1 gene product was homologous with kinesin-related protein, which may participate in chromosomal movement during meiosis, and the SRF-2 gene product was homologous with RabGAP/TBC domain-containing protein, which has GTPase-activity and may be involved in the signaling cascade in the process of spermatogenesis [21, 22]. Dioxins are suspected endocrine disruptors. Dioxin signaling cascade involving the aryl hydrocarbon receptor or dioxin receptor is well known [11]. Some dioxin responsive genes are involved in dioxin signaling cascade. The gene expression of dioxin-induced nuclear factor (DIF-3) was up-regulated [13], and the wings apart-like (WAPL) gene of *Drosophila melanogaster*, which is involved in spermatogenesis in mammals, and the SRF-2 gene, which we cloned, were down-regulated by dioxin administration [7, 22]. Gene expression of molecular chaperone Hsp20 was not changed significantly by the TCDD treatment (Fig. 3). The protein expression of Hsp27, a member of the sHSP family in the Sertoli cell line is affected by bisphenol A, a candidate of endocrine disruptor [8], and TCDD inhibits Hsp27 gene expression in mammary tumor cells [15]. Taken together, heat shock proteins are related to spermatogenesis, and the gene expression is modulated by endocrine disruptors, including dioxins. However, the

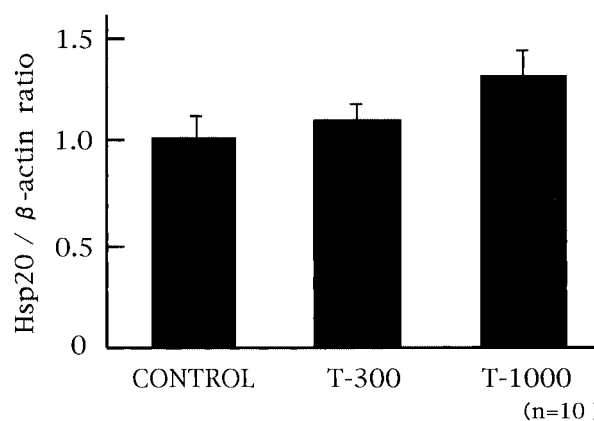


Fig. 3. Northern blot analysis was performed using 5  $\mu$ g of total RNA prepared from a TCDD-treated or non-treated 9-week-old rat testis using Hsp20 cDNA as a probe. Control rats were vehicle-injected (corn oil); T-300 group rats were TCDD-injected with a 300 ng/kg dose and T-1000 group rats were TCDD-injected with a 1,000 ng/kg dose. The mRNA expression was standardized using the intensity of the  $\beta$ -actin signal. The vertical axis is the relative radioactivity to the control sample (Hsp20/ $\beta$ -actin ratio). The ratio of the control sample was determined as 1.0. Each value represents the mean  $\pm$  SE (n=10).

expression level of all HSP members is not changed at the same time by TCDD treatment. The most plausible explanation is that Hsp20 and ODFP may be related to something such as the cytoskeleton in testes [2, 14] rather than chaperone-like activity, the expression of which may not be regu-

lated by endocrine disruptors, including TCDD, in contrast to Hsp27. Further analysis of the gene and protein expression of Hsp20 in the matured rat testis and the effect of TCDD on gene expression is required to understand the precise physiological functions of this gene product.

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