A Novel Spermatogenesis Related Factor-2 (SRF-2) Gene Expression Affected by TCDD Treatment

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Abstract. We have cloned a gene which is specifically expressed at the stage of sexual maturation in the rat testis by means of differential display, and have named it spermatogenesis-related factor-2 (SRF-2). Testicular expression was first detected at 5 weeks of age, and its level of the expression increased up to 7 weeks, and was maintained even at 63 weeks. Its cDNA was 2,789 bp in length and encoded an open reading frame of 718 amino acids. This gene was mainly expressed in the spermatocyte, judging from the result of *in situ* hybridization. The hypothetical gene product had a motif highly homologous with RabGAP/TBC protein. Taken together, this gene is considered to have some important functions for meiosis. The gene expression was significantly decreased by treatment with TCDD, a candidate endocrine disruptor, when administered to male rats of the nursing period. Body weight and testis weight were decreased by the treatment, but even then the sperm concentration in cauda epididymis was not changed significantly. SRF-2 gene may be a promising biomarker to construct a detection system of uncertain endocrine disruptors.

Key words: Endocrine disruptor, *In situ* hybridization, RabGAP/TBC protein, Spermatogenesis, TCDD

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SPERMATOGENESIS in the male, as well as oogenesis in the female, is a fundamental process for every creature, as a reproductive system to maintain its own species. The process of spermatogenesis is well characterized as the process from diploid stem cells to haploid sperm cells. In more precise terms, these cells are arranged in an array of development in the seminiferous tubules in the testis from diploid spermatogonia to haploid mature spermatids, through several stages of spermatocyte, during which process, meiosis is observed. In this complex process, the expression of numerous genes, especially those concerned with the endocrine system, is essential. Many genes involved in this process have already been cloned by differential

display [1, 2], or subtraction screening [3, 4], and thus characterized. Some of the gene products have a zinc finger motif [5–9], leucine-rich motif [10], RING finger motif [11], which are concerned with signal transduction or certain gene expression during spermatogenesis, or a WD repeat motif, which may play a role in cytodifferentiation and DNA recombination during meiosis [12].

Our goal is to construct a detection system to determine uncertain xenobiotic substances by using those spermatogenesis-related genes as biomarkers. There are many xenobiotic substances or endocrine disruptors reported so far, of which dioxins are one of the candidates. Among the dioxins, TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is one of the most toxic substances [13]. Animals were administered with TCDD throughout pregnancy and lactation, and the change in sperm number and sex accessory gland weight were observed. In the report of Faqi *et al.*, when male rats were exposed to TCDD, sperm number was decreased

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[14], but the molecular mechanism of these biological effects for the reproductive system has not to be fully clarified. It is well known that TCDD mediates its toxicity by constructing an AhR (aryl hydrocarbon receptor) and Arnt (aryl hydrocarbon receptor nuclear translocator) [15, 16] complex between TCDD, that binds to the XRE (xenobiotic responsive element) sequence, which is located upstream of the dioxin-responsive genes, such as cytochrome P450 gene family, whose expression it upregulates. Recently, the cDNA for DIF-3 (dioxin inducible factor-3), which has a zinc finger motif, was cloned from mouse testis, and the expression was induced by dioxin treatment [17]. This gene thus could be a candidate as a biomarker to assess the toxicity of dioxin for the reproductive system. As there are many genes which are concerned with spermatogenesis, the cloning and characterization of such genes are needed. Although almost all genes characterized so far are upregulated by TCDD treatment via the AhR signaling cascade, little is known about downregulated genes.

In this study, we have cloned and characterized a novel spermatogenesis-related gene (named SRF-2 gene) from rat testis by differential display, and determined its gene expression by treating rats with TCDD.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats purchased from Japan SLC Inc. were housed in an air-conditioned animal room at an ambient temperature of 22°C, a relative humidity of 55 ± 10%, and a 12-hour light/dark cycle. Animals received food and water *ad libitum*. The handling and the treatment of animals with TCDD in this experiment, described below, were performed in the hazardous chemical reagent area in our institute. All animal experiments were approved by the University of Yamanashi Animal Care and Use Committee.

Screening a SRF-2 cDNA clone from the rat testis cDNA library

A cDNA fragment, which was specifically expressed in the testis at age 7-weeks (stage of puberty) rat, compared with that of 3 weeks (nursling period) from the result of differential display (DD) [18], was cloned into

the *Sma* I site of a plasmid vector pBluescript II KS⁺ (Toyobo Biochemicals Co. Ltd., Japan). For the DD reaction, two primers were used: T₁₂GA, as an anchored primer, and OP26-13 (GTTTTTCGCAG), as an arbitrary primer. The rat testis cDNA library (Sprague-Dawley male rat testes, 10–11 weeks old, Takara Bio Inc., Japan) was screened using the cloned cDNA fragment as a probe, by colony hybridization. In brief, each colony was transferred to Hybond N⁺ nylon membranes (Amersham Biosciences Corp., NJ, USA). Probe DNA was labeled with random primer labeling system (Amersham Biosciences Corp.), using [α -³²P] dCTP. Hybridization was performed in a mixture consisting of 5 × Denhardt's solution, 5 × SSC buffer (pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 100 µg/ml denatured salmon sperm DNA, and the ³²P labeled probe DNA, at 50°C for 24 hr. The membrane was finally washed in a buffer consisting of 0.1 × SSC with 0.1% SDS at 65°C for 10 min, and was allowed to be exposed on X-ray film, which was then processed with a photographic developer (FPM100, Fuji Film Co. Ltd., Japan). The cDNA of positive clone, named SRF-2, has been analyzed so far. The DNA sequence was determined by Sanger's dideoxynucleotide chain termination method using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Co. Ltd., Japan).

Northern blotting

Total RNA from the rat testis (three to 63 weeks old), or from individual organs of seven-week old animal was prepared. Ten µg of the individual RNA was electrophoresed in a 1% agarose gel containing formaldehyde, and was transferred to a nylon membrane, Hybond N⁺ (Amersham Biosciences Corp.). The cDNA of the SRF-2 which we have cloned (accession number AB117752) was labeled with random primer labeling system (Amersham Biosciences Corp.) using [α -³²P]dCTP, and was used as a probe. Hybridization was performed, by the same procedure described in the section on colony hybridization. To confirm the integrity and the quantity of the RNA, the blot was rehybridized with rat β -actin cDNA as a probe.

In situ hybridization

The SRF-2 cDNA was subcloned into a plasmid vector, pDrive Cloning Vector (Qiagen Japan Co. Ltd.).

The inserted SRF-2 cDNA fragment, to which T7 and SP6 RNA polymerase promoters are attached at both ends, was amplified by PCR using *Taq* DNA polymerase (Nippon Gene Co. Ltd., Japan). The amplified DNA was used for riboprobe synthesis using T7 or SP6 RNA polymerase, for sense probe or antisense probe synthesis, respectively, by the Roche RNA DIG probe synthesis kit (Roche Diagnostics Co. Ltd., Japan) according to the procedure recommended by the manufacturer. From formaldehyde-fixed and paraffin-embedded tissue of eight-week old rat testes, 5- μ m thick sections were prepared for hybridization. The sections were processed on Ventana Discovery system, according to the protocol for the RiboMap *in situ* hybridization reagent system (Ventana Japan). Antisense and sense probes were hybridized respectively, and detected by anti-DIG-HRP antibody and biotin-labeled anti HRP antibody. The signals were colorized by BlueMap NBT/BCIP kit (Ventana Japan). Finally, the sections were counterstained with a nuclear fast red equivalent reagent ISH Red (Ventana Japan).

TCDD administration to rats

TCDD (AccuStandard, Germany) was preserved in toluene solution (1 mg/ml). The stock solution was diluted in corn oil (Sigma-Aldrich Co. Ltd., Japan). Maternal rats were subcutaneously injected with TCDD, dose of 300 ng/kg or 1,000 ng/kg, just after delivery, and once a week for two weeks. When offspring male rats reached three weeks old, they were weaned. Thereafter, they were injected with the same dose of TCDD, once a week, from three to eight weeks old. After the body weight was determined, they were sacrificed to remove testes at nine weeks old. Control rats were injected with equal volume of vehicle, in just the same way as TCDD administered rats. Testis weight was determined, and the sperm concentration was determined under a microscope by diluting the seminal solution 20-fold with 3% NaCl solution. RNAs from individual animals were prepared. Three μ g of RNA from each sample was used for electrophoresis, and Northern blotting was performed as described in the previous section. The intensity of each signal was standardized by using that of rat β -actin, as an internal intensity control. Results are presented as means $\pm$ SE throughout. Statistical significance was determined by Student's *t*-test.

Results

Screening a SRF-2 cDNA clone from the rat testis cDNA library

The rat cDNA library was screened by using the testis-specific cDNA fragment obtained by differential display as a probe. One positive clone was named spermatogenesis-related factor-2 (SRF-2) gene. The nucleotide sequence data of SRF-2 gene reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with an accession number AB117752. The SRF-2 cDNA was 2,789 bp in length and encoded an open reading frame consisting of 718 amino acids. The encoded region of the SRF-2 cDNA sequence was highly homologous (96%) with mouse testis specific expressing gene (accession number AK029986). The putative gene product had a homologous motif with RabGAP/TBC protein in the area between the glycine (422) and phenylalanine (628) residues. This area had a 100% identity with that of mouse KIAA protein (accession number NP_598671) (Fig. 1).

Fig. 1. The predicted amino acid sequence coded by SRF-2 cDNA sequence (I), and the amino terminus of 15 residues of KIAA protein (accession number NP_598671) are shown in alignment (II). Amino acid sequence between the 51st to the 400th coded by SRF-2 and from 16th to the C terminus of the KIAA protein is omitted in this figure. Identical amino acid residues between SRF-2 and KIAA protein are indicated by asterisks. The predicted RabGAP/TBC domain existing in the SRF-2 protein is indicated as + underneath the amino acid sequence.

Fig. 2. (A) Northern blot analysis of the RNA from various rat organs (Te: testis, Ov: ovary, Br: brain, Hr: heart, Lu: lung, Lv: liver, Kd: kidney), by using SRF-2 cDNA as probe. RNAs (10 μ g for each lane) from seven-week old rat were electrophoresed. Ribosomal RNA positions are shown on the right side as 28S (28S rRNA) and 18S (18S rRNA) with arrows, and the position of the SRF-2 mRNA signal is indicated as an arrow on the left side. To confirm the integrity and the quantity of the RNA, the blot was rehybridized with rat β -actin cDNA. (B) Northern blot analysis of the RNA from various postnatal stages of rat testis by using SRF-2 cDNA as probe. RNAs (10 μ g for each lane) from rat testis, 3, 5, 7, 10, 15, 44, and 63 weeks old were electrophoresed. The position of the SRF-2 mRNA signal is indicated as an arrow on the left side. To confirm the integrity and the quantity of the RNA, the blot was rehybridized with rat β -actin cDNA.

Time course, tissue, and cellular specific expression of SRF-2 gene

The expression of SRF-2 gene was analyzed by Northern blotting using the SRF-2 cDNA as a probe. The expression was specific to the testis, and no expression was observed in the brain, heart, lung, liver, and kidney of the seven-week old rat. The expression was not observed even in the ovary (Fig. 2A). The expression of the SRF-2 gene in the testis was observed from the five-week old rat, and was at a maximum level at week 7, and was even maintained up to 63 weeks (Fig. 2B). The SRF-2 gene expression in the seminiferous tubules was determined by *in situ* hybridization using eight-week old rat testis. Even when no signals were detected by using a DIG-labeled sense probe (Fig. 3A), purple signals were detected specifically in the cytoplasm of spermatocytes by using DIG-labeled antisense probe (Fig. 3B).

The effect of TCDD administration on male reproductive system and SRF-2 gene expression in the testis

When male rats were administered with TCDD once a week, for a total of 9 times after birth, body weight and testis weight were significantly decreased compared with those of the control group at the dose of 300 ng/kg/week and 1,000 ng/kg/week, whereas sperm

Fig. 3. Cellular localization of SRF-2 transcript in the eight weeks old rat testis. *In situ* hybridization of DIG-labeled sense (A) and antisense (B) rat SRF-2 cRNA probe to the sections of rat testis. Signals (purple color) in spermatocyte are indicated by arrows. Cells are counterstained with nuclear fast red (red color). 200 μ m size is shown in the figure by a double arrow. ST: spermatid, SC: spermatocyte.

Table 1. Effects of the exposure to TCDD on body weight (BW), testis weight (Testis WT), and sperm concentration (Sperm Conc.) in cauda epididymis

	CONT	T-300	T-1000
BW (g)	348 ± 60	288 ± 16*	283 ± 21*
Testis WT (mg)	1523 ± 83	1362 ± 112*	1353 ± 86*
Sperm Conc. (×10 ⁶ /ml)	1.20 ± 0.62	0.94 ± 0.66	0.86 ± 0.90

(n = 12, *p<0.01)

Control rats (CONT) were injected with vehicle, T-300 group rats were TCDD injected with a dose of 300 ng/kg, T-1000 group rats were TCDD injected with a dose of 1,000 ng/kg. Each value represents the mean ± SE

Fig. 4. Northern blot analysis was performed using 3 µg of total RNA, prepared from 9 weeks old TCDD treated or non treated rat testis, using SRF-2 cDNA as a probe. Control rats were vehicle injected, T-300 group rats were TCDD injected with the dose of 300 ng/kg, T-1000 group rats were TCDD injected with the dose of 1,000 ng/kg. The mRNA expression was standardized using the intensity of β-actin signal. Vertical axis is relative radioactivity to control sample (SRF-2/β-actin ratio). The ratio of the control sample was determined as 1.0. Each value represents the mean ± SE (n = 12, *P<0.01 versus control) throughout.

concentration in the cauda epididymis was not changed in the nine-week old rat (Table 1). SRF-2 gene expression was determined by Northern blotting, and was compared between the control and TCDD administered group. The gene expression was significantly decreased to about 57% in the 1,000 ng/kg/week group compared to that of the control group (Fig. 4).

Discussion

The purpose of this study was to clone and identify the spermatogenesis-related genes of mammals, to de-

termine the effect of TCDD on gene expression, and to discuss whether the gene could be used as a biomarker to assess the effect of endocrine disruptors, including TCDD, on the reproductive system. One clone, named spermatogenesis-related factor-2 (SRF-2) gene, was screened and characterized in this study. Previously, we cloned a SRF-1 gene [4] which is expressed in a similar manner as SRF-2 and was highly homologous with rat kinesin-related proteins (KRPs) [19]. The SRF-2 cDNA sequence was highly homologous with mouse testis specific expressing gene (accession number AK029986), which encodes a RabGAP/TBC domain protein [20]. On the other hand, the KIAA protein (accession number NP_598671), which is encoded by a clone in the RIKEN mouse full-length cDNA library [21], was highly homologous in part with the predicted amino acid sequence of SRF-2. The SRF-2 gene encodes a protein, which at its N terminus is 35 amino acids longer than the gene product of NP_598671. The amino acid sequence homology of the SRF-2 gene product, from the 422nd glycine to the 628th phenylalanine, had a 100% identity with that of NP_598671. The gene products of AK029986 and NP_598671 are reported to contain a RabGAP/TBC domain, which has a function of GTPase-activation, and the SRF-2 gene product also has the conserved domain (Fig. 1). This domain is considered to have some functions for membrane traffic [22], but the necessity of the domain for spermatogenesis needs to be elucidated. The expression of SRF-2 gene was specific to the testis and was observed slightly from five-week old rat, increased to the maximum level at week 7 of the stage of puberty, and was maintained at that level to 63 weeks (Fig. 2). SRF-2 gene was specifically expressed in the spermatocytes in the seminiferous tubules, judging from the results of *in situ* hybridization (Fig. 3). Taken together, this gene product may be necessary at certain critical points of meiosis in the normal spermatogenesis process.

In the process of spermatogenesis, spermatid-Sertoli cell interaction and the normal hormone production from Leydig cell are essential [23–25]. Endocrine disruptors, also called xenobiotic substances, can disturb the normal sexual hormonal system of the reproductive system of mammals. In the dioxin group, TCDD is one of the most toxic substances. TCDD signaling and the gene expression of P450 family in the hepatocyte cells are well characterized [26], but its toxicity for the reproductive system has yet to be fully clarified. In our

experiment, male rats were administered with TCDD once a week, for a total of 9 times, after birth. Body weight and testis weight were significantly decreased compared with those of the control group at the dose of 300 ng/kg/week and 1,000 ng/kg/week, whereas sperm concentration in the cauda epididymis was not changed significantly (Table 1). This result was in good agreement with the findings of other groups. In some of these experiments, TCDD was administered to the mother rats at day 15 of gestation with the dose at most of 1,000 ng/kg, and the phenotypes of the offspring male rats were observed. They observed a decreased body weight and epididymal sperm reserves, and ejaculated sperm numbers [27–29], in contrast to another group's report, which observed no change of testicular weight or sperm production [30]. In the study of Faqi *et al.*, the experimental procedure was similar to ours [14], because TCDD was administered during lactation. In other words, TCDD was administered to the mother rat prior to mating, and during pregnancy, even at the period of lactation, the dose was at most 300 ng/kg. In this experiment, sperm number per cauda epididymis was reduced in TCDD administered group, and the offspring male rats were androgen deficient as assessed by the volume of Leydig cells. Although the different results on sperm number need to be further elucidated, taken together our results were generally in good agreement with the reports of others.

Our aim was to identify a biomarker for endocrine disruptors by using spermatogenesis-related genes. For this purpose, we have compared the expression of spermatogenesis-related genes in the testis of TCDD-administered and control rats. Although the expression of the previously identified SRF-1 gene [4] was not changed significantly by TCDD treatment (data not shown), SRF-2 gene expression was decreased signifi-

cantly at the dose of 1,000 ng/kg/week group, compared with that of the control group. Body weight and testis weight were also decreased in the T-1000 group. In the experiment of Ohsako *et al.* TCDD was administered to mother rats at the gestational day 15 with the dose of 800 ng/kg at most. They observed no difference in body weight between control and 800 ng/kg dose group at postnatal day 120, but the expression of 5 α reductase type 2 and androgen receptor gene was changed by TCDD treatment in the ventral prostates of the postnatal day 49 group [30]. In our experiment, a higher dose of TCDD was administered into rats repeatedly, and it is postulated that the endocrine system of these rats were greatly affected by the TCDD administration. Taken together, the expression of SRF-2 could possibly serve as a direct marker to assess the effect of TCDD on the reproductive and endocrine system, as one of the downregulated genes by TCDD treatment.

In conclusion, we have cloned a novel spermatogenesis-related gene (SRF-2), which is expressed in the testis of mature rat. The gene could be a promising biomarker to detect uncertain endocrine disruptors including dioxin, even if the expression level is decreased by TCDD treatment. It is known that dioxins upregulate the expression of many genes, such as cytochrome P450 family genes, by the binding of the AhR/Arnt complex to an XRE sequence. As SRF-2 gene expression was downregulated by TCDD treatment, the promoter aspect of this gene should be clarified.

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