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Identification and characterization of stem cell-specific transcription of *PSF1* in spermatogenesis

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ABSTRACT

PSF1 is an evolutionarily conserved DNA replication factor, which forms the GINS complex with PSF2, PSF3, and SLD5. The mouse *PSF1* homolog has been identified from a stem cell-specific cDNA library. To investigate its transcriptional regulatory mechanisms during differentiation, we studied *PSF1* mRNA expression in testis and characterized its promoter. No canonical TATA or CAAT boxes could be found in the *PSF1* 5′-flanking region, whereas several consensus AML1, GATA, and Sry putative binding sequences are predicted within 5 kb of the putative transcription start site. In addition, binding sites for oncoproteins such as Myb and Ets were also found in the promoter. In testis, various *PSF1* gene transcription initiation sites are present and short transcripts encoding two novel isoforms, PSF1b and 1c, were found. However, spermatogonium stem cells specifically express transcripts for PSF1a. These data suggest that PSF1 is tightly regulated at the transcriptional level in stem cells.

Because of the finite life span of most mature cells, tissue stem cells, and progenitors are required to supply replacements by proliferation and differentiation. Particularly, testis and bone marrow (BM) stem cells continuously self-renew and also produce differentiated cell lineages. Hematopoietic stem cells (HSCs) and spermatogonial stem cells (SSCs) have a high regenerative capacity. In a mouse experimental model, one single HSC was found to be sufficient to reconstitute hematopoiesis when transplanted into a BM-ablated recipient [1]. When spermatogenesis is disrupted by high temperatures or drugs, surviving SSCs can regenerate spermatogenesis [2,3]. Because these tissue stem cells have great regenerative capacity for reconstituting ablated tissues, ex vivo amplification of stem cells without loss of self-renewal and multidifferentiation potential represents an important target for transplantation, gene, and cellular therapies. In order to study the molecular mechanisms regulating self-renewal of stem cells, knock-out (KO) mice lacking several cell cycle-related genes, such as p27, p18, and ATM, were generated as previously reported [4–6]. However, their downstream function in the self-renewal process, especially regarding molecules involved in DNA replication, is currently not known.

The initiation of DNA replication in eukaryotic cells is mediated by a highly ordered series of steps involving multiple complexes at replication origins [7,8]. This process commences with the binding of the origin recognition complex (ORC) to replication origins. CDC6 and Cdt1 bind to ORCs to act as loading factors for the Mcm2-7 (minichromosome maintenance) complex and then pre-replication complexes (pre-RC) are established. At the G1/S transition of the cell cycle, the pre-RCs are transformed into initiation complexes (ICs). Activation of MCM helicase activity requires the action of two protein kinases, DDK (Cdc7-Dbf4) and CDK (cyclin-dependent), as well as the participation of at least eight additional factors, including Mcm10, Cdc45, Dpb11, synthetic lethal with dpb11 mutant-2 (Sld2), Sld3, and GINS [9]. GINS was recently identified as a novel heterotetrameric complex from lower eukaryotes. It consists of four subunits, SLD5, PSF1, PSF2, and PSF3, each of approximately 200 amino acid residues highly conserved in all eukaryotes and essential for both the initiation and progression of DNA replication [10–12].

By using lower eukaryote models, multiple steps for progression of DNA replication are now well understood at the protein level, e.g. phosphorylation, degradation, and/or interaction processes; however, how these factors are activated or inactivated at the RNA level in mammalian tissues consisting of multiple cell lineages and cells in different phases of the cycle has not been elucidated. Previously, we cloned the mouse ortholog of *PSF1* and *SLD5* from an HSC-specific cDNA library or by two-hybrid screening of a cDNA library derived from embryos [13,14]. Transcription of *PSF1* is predominantly found in highly proliferative tissues, such as BM and testis. Loss of *PSF1* causes embryonic lethality around the implantation stage [13], with *PSF1*$^{-/-}$ embryos showing impaired proliferation of multipotent stem cells, i.e., the inner cell mass. However, the transcriptional regulation of *PSF1* in immature cells is not understood.

Abbreviations: GINS, Go-ichi-nii-san; PSF, partner of SLD5; SSCs, sperm stem cells; HSCs, hematopoietic stem cells

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Here, we have isolated the 5′ promoter sequence of the *PSF1* gene to investigate regulatory mechanisms responsible for tissue-specific expression. We report an in silico analysis of the potential *cis*-regulatory elements in the *PSF1* promoter. In addition, we studied SSC-specific transcription initiation sites and multiple transcription initiation sites of *PSF1* in testis.

Materials and methods

Animals. ICR mice were purchased from Japan SLC (Shizuoka, Japan). All animal studies were approved by the Osaka University Animal Care and Use Committee.

In situ hybridization. cDNA fragments were amplified by PCR using the primer set 5′-GAA TTC AAA GCT TTG TAT GAA CAA AAC CAG-3′ and 5′-GTC GAC TCA GGA CAG CAC GTG CTC TAG AAC T-3′, followed by ligation into pT7 Blue Vector (Novagen Inc., Madison, WI, USA). These plasmids were used for probe synthesis. Antisense and sense cRNA probes were synthesized using digoxigenin (DIG)-RNA labeling kits with T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN, USA). Hybridization was performed as previously described [15].

Cell culture. Colon 26 cells were maintained in DMEM medium containing 10% FBS, 100 IU penicillin and 100 μg/ml streptomycin. The cells were plated in 10 cm tissue culture dishes in 5% CO_2 and 95% air at 37 °C.

5′-Rapid amplification of cDNA ends (5′-RACE). 5′-RACE was performed as described previously [16]. In brief, we used the 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was purified from whole testis, embryonic day (E) 10.5 embryos, colon 26 cells, and sorted cells (see below) by the guanidine-thiocyanate extraction method. Total RNA of sorted cells was purified using RNeasy Plus Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. *PSF1* transcripts were reverse-transcribed with Superscript II (Invitrogen) using the gene-specific primer PSF1GSP1 5′-GCA TGT CTG TCA ATT TAA-3′. The products were poly(C) tailed by terminal deoxynucleotidyl transferase and amplified by PCR, using an anchor primer and the gene-specific primer 5′-CGA AGC AAC CGG TCA TAC A-3′ (PSF1ANGSP1). The amplified products were subcloned into pT7 Blue vector (Novagen).

5′ End amplification of PSF1 cDNA using 5′ End oligo-capped cDNA library. CapSite cDNA (Nippon Gene., Toyama, Japan) from mouse testis and embryo (Day15) was used as a template for PCR with primer 1RC (Nippon gene., Toyama, Japan) corresponding to the oligoribonucleotide sequence ligated at the cap site and a *PSF1* gene-specific primer, 5′-CGA AGC AAC CGG TCA TAC A-3′ (PSF1ANGSP1) and the PCR product was then used as a template for the second round PCR with nested primers 2RC (Nippon Gene) and 5′-GCTATCGTGCAGCGTCTATT-3′ (PSF1ANGSP2). The amplified products were used for Southern blotting or purified, cloned and sequenced.

Southern blotting. Amplified 5′-ends of cDNA by 5′-RACE (see above) were separated on 0.8% agarose gels and transferred onto nylon membrane filters which were hybridized overnight at 60 °C in DIG Easy Hyb (Roche Diagnostics, Germany) with a digoxigenin-labeled *PSF1* cDNA probe. The hybridized probe was detected with alkaline phosphatase-conjugated antidigoxigenin antibodies using the DIG luminescent detection kit (Roche), following the manufacturer's instructions. The probe was prepared by using PCR DIG Probe Synthesis Kits (Roche) with primers: E11prb-2s (5′-AGC TGG TTG CTG GTG TTG TGC GAC-3′) and E11prb-2r (5′-CGA AAA CAA GAA ACG CTC AGA TGG G-3′).

Enrichment of SSCs. Isolation of SSCs was as previously reported [17]. Briefly, experimental cryptorchid testes were produced by suturing the testis fat pad to the abdominal wall at 7 weeks of age. After 2 or 3 months, testes were dissected and then digested with collagenase (Type IV, Sigma, St Louis, MO) at 32 °C for 15 min. The cells were next digested with DNase (Sigma) and trypsin (GIBCO) at 32 °C for 10 min. When most of the cells were dispersed, the action of trypsin was terminated by adding PBS containing 1% fetal bovine serum. Cell sorting was performed as described previously [18]. The antibody used for detection of SSCs was a FITC-conjugated anti-α6-integrin antibody (BD Biosciences. San Jose, CA). Control cells were stained with isotype-matched control antibodies. After the final wash, cells were resuspended in 2 ml of PBS/FBS containing 1 μg/ml propidium iodide for identification of dead cells. The stained cells were analyzed by FACS Calibur (Becton & Dickinson, New Jersey, USA), and sorted by JSAN (Bay Bioscience, Kobe, Japan).

Results and discussion

Proliferating germ cells express high levels of PSF1 transcripts

Previously we reported that *PSF1* transcripts are highly expressed in testis and BM [13]. In addition, immunohistochemistry also revealed the presence of PSF1 protein specifically in the spermatogonia, lining the outermost layer of the testis [13]. Here, to study the expression of *PSF1* mRNA in the testis, we performed in situ hybridization with an antisense-probe which hybridized to spermatogonia (arrow) and spermatocytes (arrowhead) (Fig. 1A). No obvious signals were recorded when using the sense-probe (Fig. 1B). These data indicate that stem cell-specific expression of PSF1 may be regulated at the transcriptional or post-transcriptional level, because although PSF1 protein could be detected in the spermatogonia this was not the case in spermatocytes [13].

Identification and characterization of PSF1 transcription initiation sites

In order to identify the *PSF1* promoter, we performed 5′-RACE analysis with *PSF1* gene-specific primers. Total RNA from testis, colon 26 cells, and whole embryo were reverse-transcribed (see Materials and methods). Although a single DNA band <372 bp in length was obtained after nested PCR of colon 26 and whole embryo, a broad band was amplified from testis cDNA (Fig. 1C,a). We also observed smaller transcripts in 5′ End amplificated cDNA of *PSF1* using 5′ End oligo-capped cDNA library from testis compared to that from embryo (Fig. 1C,b). To exclude that this was caused by degraded RNA in our sample, total RNA was visualized by Ethidum bromide (Fig. 1D). The quality of the RNA in each sample was essentially the same. Thus, the broad band seen in testis was not caused by RNA degradation. We then amplified each cDNA fragment, purified them and subcloned them for DNA sequencing. Here, the 5′-end of the longest cDNA fragment is defined as "+1" (Table 1, +1 5′-TGC ACT TCT ATT-3"). It was located 157 bp upstream of the first ATG.

Putative cis elements of the 5′-flanking regions of PSF1

To characterize the 5′-flanking region of PSF1, we analyzed putative transcription binding sites in silico (Fig. 3). The proximal 5′-flanking region of the *PSF1* gene lacks consensus CAAT or TATA boxes, but possesses consensus Sp1 binding sites, which are characteristic of TATA-less gene promoters [19]. In the 5′-flanking region of *PSF1*, several putative *cis*-acting elements were identified, such as E2F, GATA, Myb, AML1, Evi-1, and Sry.

As previously reported, E2F is known to regulate DNA replication, cell cycle progression, DNA repair, and differentiation