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Restricted Expression of a Member of the Transcription Elongation Factor S-II Family in Testicular Germ Cells during and after Meiosis[1]

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Whether expression of transcription elongation factors is regulated during development has not been investigated, though genes encoding elongation factor S-II are transcribed in a tissue-specific manner. We investigated the expression profile of tissue-specific S-II during development using an isolated cDNA, termed mouse *S-II-T1*, whose transcripts are detected almost exclusively in testis. Three experiments were performed with various types of germ and somatic cells in testis to determine in which cells *S-II-T1* is expressed. (1) Expression of *S-II-T1* is markedly reduced in the testes of adult WBB6F1-W/W^v mutant mice which lack testicular germ cells, indicating its expression is specific to testicular germ cells. (2) The onset of mouse *S-II-T1* mRNA appearance in testis is seen about 10-14 days after birth, which is consistent with the start of meiotic events, suggesting that *S-II-T1* is not transcribed in premeiotic and early meiotic cells such as spermatogonia, leptotene spermatocytes, or zygotene spermatocytes. (3) Mouse *S-II-T1* transcripts accumulate in meiotic pachytene spermatocytes and are detected in postmeiotic haploid cells such as round and elongated spermatids during spermatogenesis, as shown by fractionation of testicular germ cells at four different stages. These results indicate that expression of mouse *S-II-T1* is restricted to testicular germ cells during and after meiosis in the course of spermatogenesis. This is the first report that expression of a transcription elongation factor in particular cells is regulated in a stage-specific manner in the course of development.

Key words: arrest-relief, development, pausing, RNA polymerase II, spermatogenesis.

Regulation of gene expression at the level of transcription elongation is observed in many genes including histone H3.3, adenosine deaminase, c-*myc*, c-*myb*, and c-*fos* and numerous viral genomes (*1-9*). S-II is a eukaryotic factor involved in the process of transcriptional elongation, which was initially purified by Natori and coworkers as a factor that stimulates the transcriptional activity of RNA polymerase II *in vitro* (*10-14*). S-II has been implicated in the process of transcriptional pausing, mainly through analyses using adenovirus and histone H3.3 genes as templates. S-II promotes transcription by RNA polymerase II against the inhibitory effects of elongation on these genes (*15-21*). This activity is interpreted as facilitating nascent transcript cleavage within the paused RNA polymerase II in the elongation complex (*22-27*).

Recently, different isotypes of *S-II* have been discovered and shown to be expressed in restricted tissues (*28-30*). However, the functional significance of S-II in the regulation of genes which are expressed in a tissue-specific manner during development or differentiation has not been answered. Though the tissue-specific activities of these S-II family members are not yet understood, it is possible that expression of particular genes or gene families could be regulated by such tissue-specific S-II in the process of transcriptional elongation. It is thus important to analyze the expression profiles of such tissue-specific *S-II* to determine the targeted cells and genes in which they are utilized, and to further resolve the molecular mechanisms by which they function.

In this paper, we describe the expression profile of mouse testis-specific *S-II* (*S-II-T1*) in the course of spermatogenesis. *S-II-T1* mRNA levels were investigated; (1) with the testes of mice deficient in germ cells, (2) during sexual maturation, and (3) using fractionated testicular germ cells. Transcripts of mouse *S-II-T1* were found to be accumulated specifically in pachytene spermatocytes and haploid cells, but not in somatic cells such as Sertoli cells nor premeiotic spermatogonia.

MATERIALS AND METHODS

Animals—Male ICR mice (Nippon Bio-Supp. Center, Tokyo) were used for all RNA preparations and analyses except Northern blotting using the testes of mutant mice.

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Eight-week-old WBB6F1-W/W^v and control WBB-6F1+/+ litter mates for the mutant analysis were purchased from the Shizuoka Experimental Animal Farm.

Preparation of RNA—Total RNA was prepared by the acid guanidine/phenol/chloroform (AGPC) method essentially as described by Chomczynski and Sacchi (*31*). Poly(A)$^+$RNA was isolated by using Oligotex-dT30 (Takara Shuzo, Kyoto) according to the manufacturer's recommendations.

Isolation of Mouse S-II-T1 cDNA—Partial cDNAs encoding S-II family members were screened from mouse poly(A)$^+$RNA using reverse transcription polymerase chain reaction (RT-PCR), with two forward primers, 5′-AA(C/T)ACIGA(C/T)ATGAA(A/G)TA(C/T)AA-3′ (sense 1) and 5′-GA(A/G)GA(A/G)ATGGCI(A/T)(G/C)IGA(C/T)GA-3′ (sense 2); and the reverse primer, 5′-TTCCAIC(G/T)(A/G)TTICC(A/G)CA(C/T)TC-3′ (antisense). These correspond to +538 to +557, +670 to +689, and +871 to +890 of human *S-II-T1* (*30*), respectively. Poly(A)$^+$RNAs isolated from brain, liver, skeletal muscle, spleen, kidney, testis, and embryo were used as templates, respectively. RT-PCR was performed under the following conditions: 1.5 min at 95°C, 2 min at 37°C, or 42°C, 3 min at 63°C for 30 cycles. The amplified products of the expected sizes were cloned into the *Sma*I site of pBluescript II SK(−) (Stratagene) and sequenced by the dideoxy-sequencing method using custom oligo primers. The nucleotide sequence of one cDNA showed similarity to that of testis-specific *S-II* (*29, 30*). Two other cDNAs had sequences identical to known *S-II* sequences (*28, 32*). The fragments which were conserved with testis-specific *S-II* sequences were labeled with [^{32}P]dCTP by random priming (Boehringer Mannheim) to screen a mouse erythroleukemia cell λZAPII cDNA library, kindly provided by Drs. M. Oishi and T. Watanabe. One positive clone containing an approximately 1.2-kb insert was isolated from 1×10^6 plaques. The insert fragment (pmSIIT1) was obtained by excision with a helper phage, followed by dideoxy-sequencing of both strands of pmSIIT1 to determine its primary structure.

Preparation and Separation of Testicular Germ Cells—Testes were dissected from 20 adult (8-week-old) male mice and the tunic albuginea was removed in PBS containing 0.1% glucose, using forceps. The seminiferous tubule masses were incubated in PBS containing 0.1% glucose and 0.1% collagenase S1 for 20 min with gentle shaking in a water bath at 33°C. The dissociated semiferous tubules were washed several times with PBS containing 0.1% glucose, resuspended in PBS containing 0.1% glucose, 200 U/ml of trypsin, and 120 U/ml DNaseI and incubated for 10 min with vigorous shaking in a water bath at 31°C. The suspension was placed on ice, 8% fetal calf serum, 0.1% soybean trypsin inhibitor, and 500 U/ml of DNaseI were added and the suspension was filtered with glass wool and Falcon Cell Strainer 2350 (70 μm) to remove aggregates. All steps in cell separation and fractionation were carried out at 4°C.

Centrifugal elutriation of mouse testicular germ cells was carried out with a Beckman JE6 elutriation rotor essentially as described by Meistrich *et al.* (*33*).

RNA Analysis—RNA was electrophoresed on a 1.5% agarose-formaldehyde gel and transferred to a nylon filter (Gene Screen plus; NEN). Filters for Northern blot analysis were hybridized at 42°C in 50% formamide/10×Denhardt's solution/5×SSPE/2% SDS/100 μg/ml salmon sperm DNA for 12-16 h using the pmSIIT1 *Eco*RI/*Xho*I fragment labeled with [^{32}P]dCTP. Filters were subsequently washed three times at room temperature in 2× SSC for 10 min and then twice at 50°C in 2×SSC/0.1% SDS for 20 min. Exposure was continued for 1-5 days. The same procedures were performed using human β-actin cDNA (Clontech) as a control probe.

RESULTS

The Primary Structure and Characteristics of Mouse S-II-T1—We isolated three different partial cDNAs encoding mouse S-II family members by RT-PCR using several degenerated primers and poly(A)$^+$RNA from various tissues. Two of them were identical to known mouse *S-II* (*28, 32*) while one did not match the known sequences. Figure 1A shows the nucleotide and deduced amino acid sequences of the full-length cDNA (named mouse *S-II-T1*) isolated by plaque hybridization using the RT-PCR product as a probe. Mouse S-II-T1 is closely related to the testis-specific human and rat S-II-T1 with 89 and 99% sequence identity in overall primary structure, respectively (Fig. 1B).

Specific Distribution of Mouse S-II-T1 mRNA in Testicular Germ Cells—Northern blot analysis of RNA prepared from multiple tissues showed expression of mouse *S-II-T1* to be highly restricted to testis (Fig. 2A). Testis is known to consist of a variety of germ cells, and somatic cells such as Sertoli cells, Leytig cells, and macrophages. To elucidate whether expression of *S-II-T1* in testis is restricted to germ or somatic cells, we compared *S-II-T1* transcripts in the testes of the WBB6F1-W/W^v mutant with those of WBB6F1+/+ wild-type mice. The testes of adult WBB6F1-W/W^v mice are known to contain somatic cells, but lack testicular germ cells because of a mutation in the c-*kit* gene (*34*), thus enabling us to distinguish whether expression of particular genes is specific to male germ cells. The *S-II-T1* transcripts are hardly detected in the testes of adult WBB6F1-W/W^v mutant mice (Fig. 2B, lanes 2 and 4) while abundant *S-II-T1* transcripts exist in those of wild-type mice (Fig. 2B, lanes 1 and 3). β-Actin gene transcripts in the testes of the WBB6F1-W/W^v mutant and those of wild-type mice are almost equivalent. Though we can not exclude the possibility that the lack of *S-II-T1* transcripts in testicular somatic cells of WBB6F1-W/W^v mice is due to some interference with normal development of these cells by the mutation, these results strongly indicate that expression of mouse *S-II-T1* is specific to testicular germ cells.

Expression Profiles of Mouse S-II-T1 Transcripts during Sexual Maturation—As testicular germ cells differentiate dramatically through a variety of stages during spermatogenesis, it is necessary to determine in which type of germ cells S-II-T1 is expressed. We investigated the expression profiles of mouse *S-II-T1* mRNA during sexual maturation. Poly(A)$^+$RNA loaded are almost equivalent from 4 to 56 days of age since transcripts encoding the β-actin gene are distributed almost equally during the corresponding periods. *S-II-T1* transcripts are scarcely visible in the testes of 4, 7, and 10-day-old mice (Fig. 3, lanes 1-3). Mice within 10 days after birth are known to have in their testes premeiotic spermatogonia, early meiotic germ cells such as

leptotene spermatocytes and zygotene spermatocytes, but do not have meiotic pachytene spermatocytes nor post-meiotic haploid cells (*35, 36*). Therefore, the mouse *S-II-T1* gene is not transcribed in premeiotic or early meiotic testicular germ cells. Accumulation of *S-II-T1* mRNA begins around 10–14 days after birth (Fig. 3, lanes 3 and 4), corresponding to the developmental emergence of pachytene spermatocytes in the testes, which indicates that mouse *S-II-T1* transcripts seem to be expressed at least in pachytene spermatocytes. The onset of up-regulation of the mouse *S-II-T1* gene is also consistent with the start of meiosis in the course of spermatogenesis. The proportion of

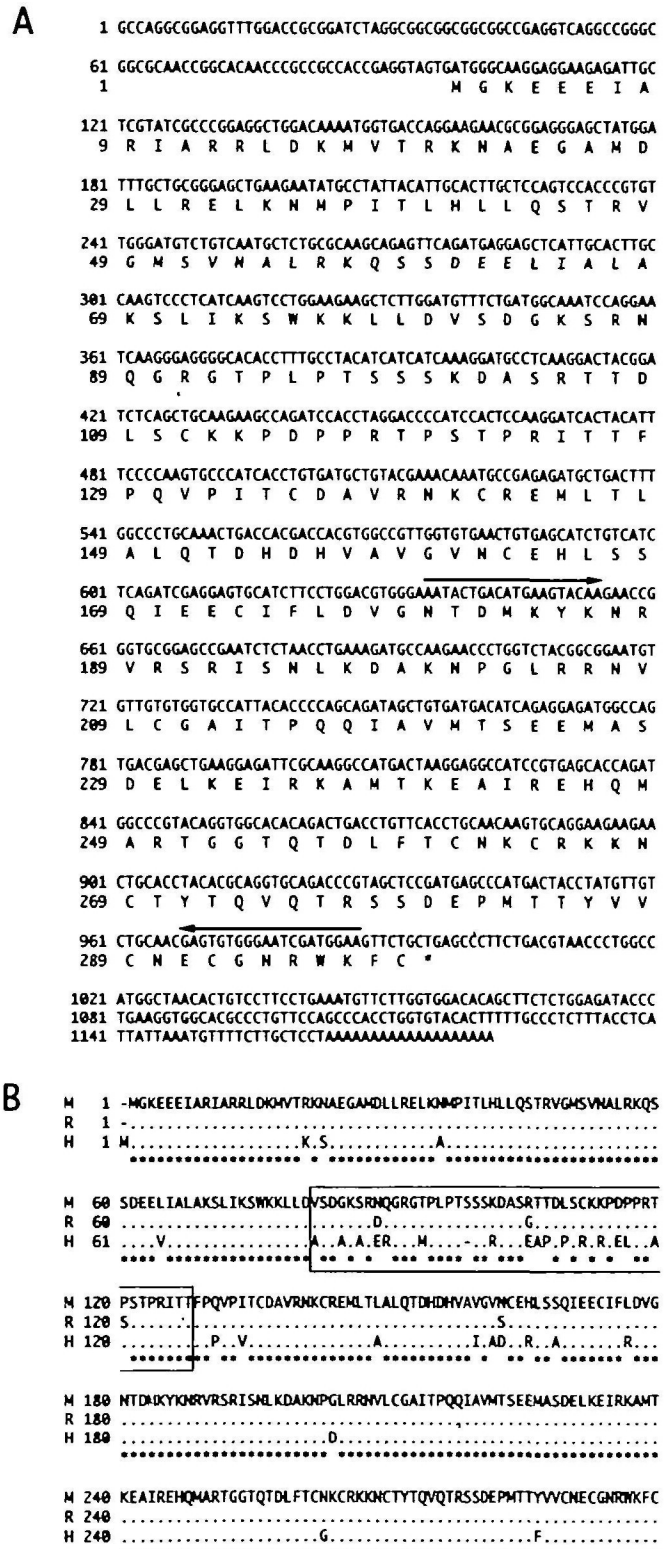

Fig. 1. **Isolation of a mouse cDNA encoding the S-II-T1 family member.** (A) The nucleotide sequence of the cDNA for mouse *S-II-T1* and its deduced amino acid sequence. The sequence for mouse *S-II-T1* consists of an open reading frame of 897 nucleotides and encodes a protein of 299 amino acids. Numbers indicate the positions of nucleotides (upper) or amino acids (lower) on the left side. Primers used for RT-PCR are shown as arrows. The DDBJ accession number of this sequence is D86081. (B) Amino acid alignment of S-II-T1 family members. M, R, and H indicate the amino acid sequences of mouse, rat, and human S-II-T1, respectively. Asterisks indicate the conserved amino acids among these S-IIs. Numbers indicate the positions of amino acids to the left. A box represents the region which is not conserved with ubiquitous S-II (*32*).

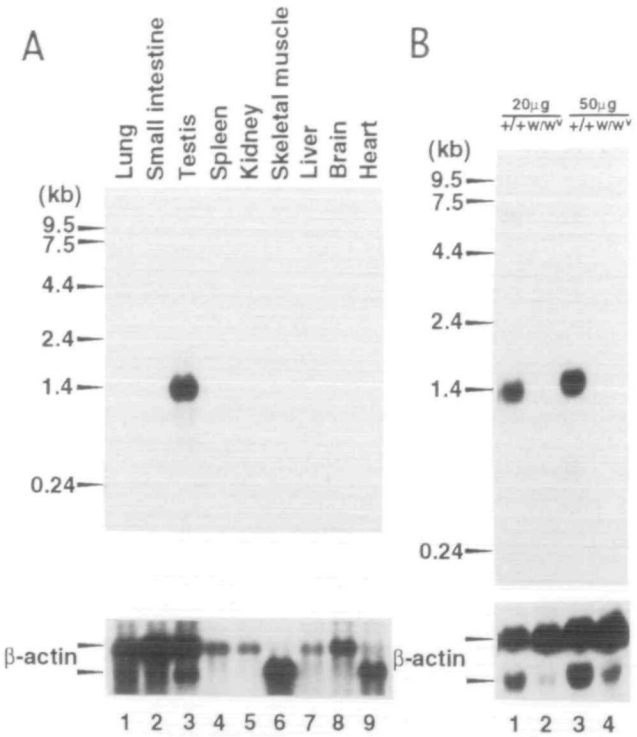

Fig. 2. **Restricted expression of mouse *S-II-T1* mRNA in testicular germ cells.** (A) Tissue-distribution of mouse *S-II-T1* mRNA. Full-length mouse *S-II-T1* cDNA (upper) and human β-actin cDNA (lower) were used as probes. The estimated size of mouse *S-II-T1* transcripts was approximately 1.4 kb, which is almost identical to those of human and rat *S-II-T1* but distinct from that of mouse ubiquitous *S-II* (data not shown). The positions of size markers and β-actin transcripts are indicated by arrowheads to the left. Poly(A)$^+$ RNA used for analysis was harvested from lung (lane 1), small intestine (lane 2), testis (lane 3), spleen (lane 4), kidney (lane 5), skeletal muscle (lane 6), liver (lane 7), brain (lane 8), and heart (lane 9) of 8-week-old mice. (B) Comparison of mouse *S-II-T1* mRNA accumulation in the testes of adult WBB6F1+/+ and WBB6F1-*W*/*W*^{v} mutant mice. Twenty micrograms (lanes 1 and 2) and 50 μg (lanes 3 and 4) of total RNA harvested from the testes of adult WBB6F1+/+ (lanes 1 and 3) and WBB6F1-*W*/*W*^{v} mutant mice (lanes 2 and 4) were loaded.

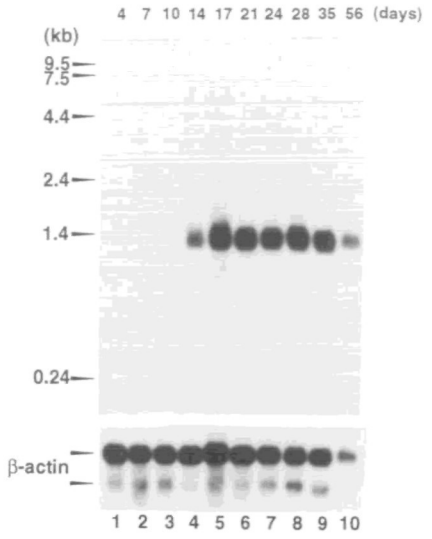

Fig. 3. ***S-II-T1* mRNA expression level during sexual maturation.** The age is listed above the autoradiograms as an indicator of the stage of sexual development. Ages of mice used for analysis were: 4 (lane 1), 7 (lane 2), 10 (lane 3), 14 (lane 4), 17 (lane 5), 21 (lane 6), 24 (lane 7), 28 (lane 8), 35 (lane 9), and 56 (lane 10) days after birth. Each lane contains 2 μg of poly(A)$^+$RNA. The positions of size markers and β-actin transcripts are indicated by arrowheads to the left.

pachytene spermatocytes in mouse testis reaches its maximum at around 17 days of age and then decreases during sexual maturation. However, the mRNA level of *S-II-T1* is almost constant from the testes of 17-day-old mice to those of adult (56-day-old) mice (Fig. 3, lanes 5–10), suggesting that mouse *S-II-T1* transcripts exist also in postmeiotic germ cells.

Expression Profiles of Mouse S-II-T1 Transcripts in Fractionated Testicular Germ Cells—To analyze whether the accumulation of S-II-T1 transcripts continues through postmeiotic germ cells such as round and elongated spermatids, we performed a fractionation of mouse testicular cells by centrifugal elutriation. This method enabled us to separate distinct cells in accordance with their size. We obtained different cell fractions enriched in pachytene spermatocytes, round spermatids, elongated spermatids, and residual bodies, respectively (Fig. 4). Northern blot analysis showed *S-II-T1* transcripts to be accumulated in the fraction of pachytene spermatocytes but to also be detected in the fraction enriched in round and elongated spermatids and residual bodies (Fig. 5), while the amounts of β-actin transcripts including subtypes and those of 28S rRNA (data not shown) were roughly equivalent among the fractions. The fractions rich in pachytene spermatocytes and two distinct spermatids contain small amounts (no more than 5%) of somatic Sertoli cells and mitotic spermatogonia, respectively, as shown in Fig. 4, b–d. However, few *S-II-T1* transcripts seem to be expressed in Sertoli cells and spermatogonia (Figs. 2 and 3), suggesting that signals detected in the fractions enriched in pachytene spermatocytes and both spermatids (Fig. 5, lanes 1–3) are attributable to the expression of *S-II-T1* in pachytene spermatocytes and both spermatids, respectively. The observation that pachytene spermatocytes are not included in the fractions of round and elongated spermatids (Fig. 4, c and d) indicates that *S-II-T1* transcripts also exist in haploid cells (Fig. 5, lanes 1–3). We thus conclude that the accumulation of *S-II-T1* mRNA is specific to testicular germ cells during and after meiosis and does not appear in premeiotic and early meiotic germ cells or in somatic cells in the course of spermatogenesis, as summarized in Fig. 6.

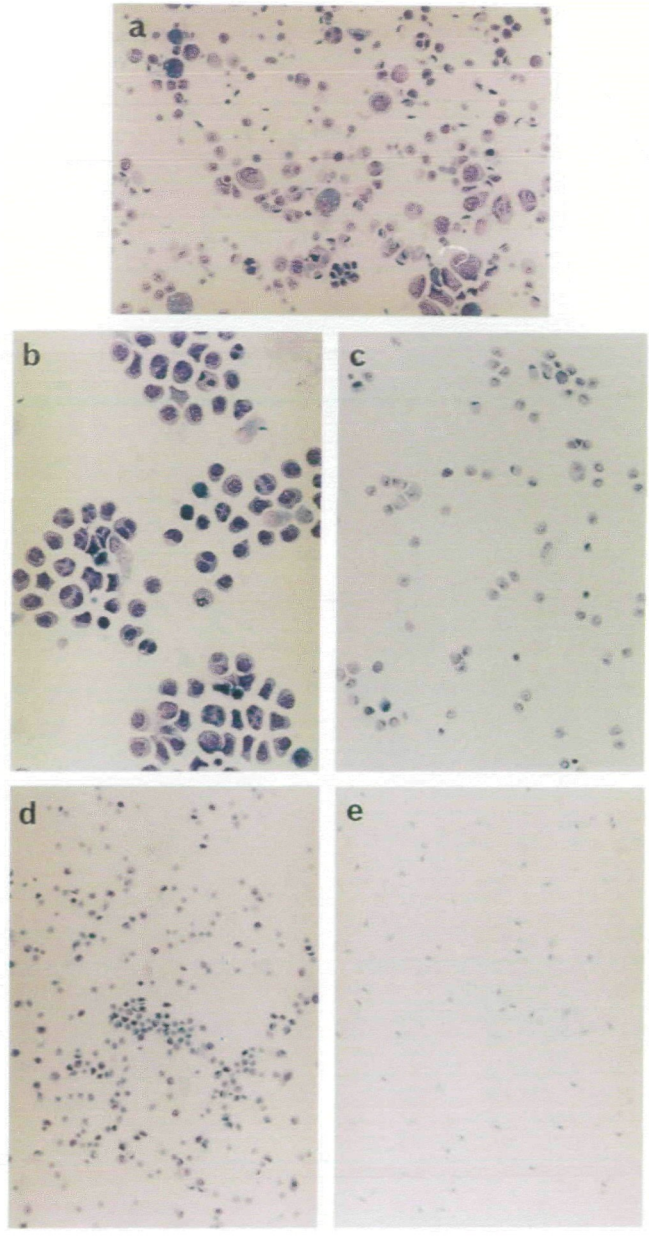

Fig. 4. **Phase-contact microscopic appearance of fractionated mouse testicular germ cells at four different developmental stages.** Mouse testicular cells before fractionation (a); fraction enriched in: pachytene spermatocytes (b); round spermatids (c); elongated spermatids (d); residual bodies (e).

DISCUSSION

cDNAs encoding tissue-specific S-II have been identified, though their specific function remains unknown. It is possible that the expression of these *S-II* family members is regulated specifically in the process of differentiation

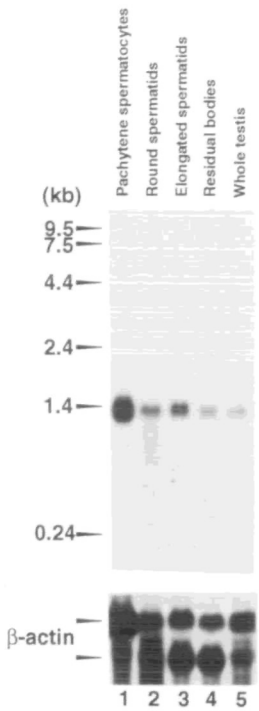

Fig. 5. **Accumulation of mouse *S-II-T1* mRNA at different developmental stages of testicular germ cells.** Twenty micrograms of total RNA were blotted onto the membrane. RNAs were prepared from: pachytene spermatocytes (lane 1); round spermatids (lane 2); elongated spermatids (lane 3); residual bodies (lane 4); whole testis (lane 5). The positions of size markers and β-actin transcripts are indicated by arrowheads to the left.

and/or development. Therefore, we analyzed the expression profiles of *S-II-T1*, a testis-specific *S-II*, in the course of spermatogenesis and obtained data which indicated that this S-II appears only during restricted stages of spermatogenic cells. This was the first evidence showing that the expression of a transcription elongation factor in particular cells is regulated in a stage-specific manner during development. Mouse *S-II-T1* was shown to be transcribed initially in meiotic pachytene spermatocytes and not in mitotic spermatogonia or testicular somatic cells. This suggests that mouse S-II-T1 is required for meiotic events, probably through the transcriptional regulation of particular genes at the level of elongation. We also demonstrated that *S-II-T1* transcripts exist in haploid cells as well as in pachytene spermatocytes. During preparation of this manuscript, Natori and coworkers have reported the results of *in situ* hybridization analysis by using a preliminary partial sequence of mouse *S-II-T1* and concluded that the expression of *S-II-T1* is restricted to spermatocytes (*37*). However, the images they presented do not allow discrimination of different spermatogenic cells, and one can not distinguish whether or not there are spermatids in the sections used. As our Northern blot analysis of the expression profiles of mouse *S-II-T1* was carried out by handling a cell fraction of spermatids which did not contain pachytene spermatocytes, we concluded that *S-II-T1* transcripts must exist in haploid cells as well as in pachytene spermatocytes, though the mRNA level seems to be lower than in pachytene spermatocytes (Fig. 6). Further investigation of the S-II-T1 protein (amount, extent of phosphorylation

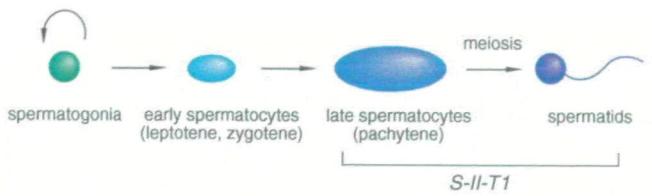

Fig. 6. **Summary of expression profiles of *S-II-T1* mRNA during spermatogenesis.** The spermatogenic cells in which *S-II-T1* mRNA is accumulated are shown.

etc.) in spermatogenic cells will be necessary to determine its potential function in these cells since the genes expressed in the testis often undergo translational regulation.

The expression profiles of mouse *S-II-T1* in male germ cells resemble those of testis-specific histones which are transiently utilized for packaging genomic DNA during spermatogenesis (*38-41*). Taking into consideration the fact that the histone H3.3 gene has intrinsic pausing sites of RNA polymerase II which can be read through by ubiquitous S-II (*6, 17*), S-II-T1 might have activities to relieve arrest by the putative termination sites within the genes of testis-specific histones in a specific manner. Mouse *S-II-T1* is also detected at stages after meiosis, though the amount of transcripts in postmeiotic cells seems to be less than in pachytene spermatocytes, suggesting that S-II-T1 could regulate transcriptional elongation of genes which are transcribed only in spermatids such as genes for protamines and transition proteins (*42, 43*).

In conclusion, we demonstrated that expression of *S-II-T1* is specific to testicular germ cells during and after meiotic stages. These types of cells are known to differentiate into mature sperm, expressing particular genes that encode proteins required in this spermatogenic process. Thus, characterization of the expression profile of *S-II-T1* makes it possible to converge potential candidate genes whose expression can be regulated by this S-II. We think that the results presented here denote the beginning of an understanding of the regulatory mechanisms of transcriptional elongation during development. Functional analyses of S-II-T1 upon transcriptional elongation of genes expressed specifically during and after meiosis will help us elucidate the potential roles of stage and/or tissue-specific S-II. Moreover, isolation of the mouse genomic DNA encoding S-II-T1 will make it possible to analyze the *in vivo* function of this S-II during spermatogenesis using gene-targeting methods. Further biochemical and genetical analyses are required for understanding the molecular mechanisms in which S-II-T1 takes part during spermatogenesis.

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