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Expression of Cyclin D3 Transcripts in the Postmeiotic Male Germ Cells of the Mouse

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D-type G1 cyclins are known to be crucial for the progression of mitotic cell cycle in mammals. Although many studies have been performed to elucidate the roles of D-type cyclins, it is largely unknown whether D-type cyclins are directly involved in the regulation of meiotic germ cell development. In the present study, we examined the expression patterns of D-type cyclins (cyclin D1 and D3) during male germ cell development by northern blot and *in situ* hybridization analyses. In the adult testes, we detected a 4.2 kb cyclin D1 mRNA and two different sizes (2.3 kb and 1.8 kb) of cyclin D3 mRNAs. The short form of the cyclin D3 transcript was testis-specific. Along with the testicular development, expression of cyclin D3 mRNA was increased whereas cyclin D1 mRNA was gradually decreased. *In situ* hybridization study also revealed that the expression of cyclin D3 was restricted to the postmeiotic germ cells. Furthermore, the 2.3 kb transcript was highly expressed in the round spermatids and decreased in the elongated spermatids/residual bodies, while the 1.8 kb transcript was expressed in elongated spermatids/residual bodies more abundantly. Sucrose-gradient separation of polysomal RNA fractions demonstrated that some portions of the 2.3 kb transcript are translationally active, while the 1.8 kb transcript is likely to be inactive. Taken together, the present data suggest a functional importance of cyclin D3 expression in the differentiated postmeiotic male germ cells.

Cyclins together with their catalytic counterparts, cyclin dependent kinases (cdk), are critical for normal development and differentiation in eukaryotes (for review, see Sherr, 1993). Among various cyclins, G1 cyclins characteristically appear during the G1-phase of the cell cycle. To date, more than eight kinds of G1 cyclins have been identified and classified into three categories, C, D, and E. The D-type cyclins are encoded by a family of three closely related genes, cyclin D1, D2 and D3 (Sherr, 1993). Several lines of evidence suggest that cyclin D1 is the rate-limiting molecule for passage through the G1-phase. For example, treatment with antibodies against cyclin D1 blocks the entry of the S-phase in human fibroblasts, and overexpression of cyclin D1 shortens the G1 period (Baldin et al., 1993; Lukas et al., 1994). Since overexpression of cyclin D1 in serum-starved cells does not result in S-phase entry, it appears that the expression of cyclin D1 is necessary, but not sufficient for the progression of G1- to S-phase (Resnitzky et al., 1994). Moreover, cyclin D1 was originally identified in the context of the delayed early response to growth factor stimulation (Matsushime et al., 1991). Therefore, cyclin D1 is believed to be a primary growth-signal responder in many cell types rather than the regulator of the G1/S transition (Sherr, 1993). The precise roles of other members of D-type cyclins, cyclin D2 and D3, are not clear. D-type cyclins are expressed in a highly cell type-specific manner and appear sequentially in the G1-phase, suggesting that these D-type cyclins may have distinct roles in the cell cycle regulation, although redundant functions cannot be excluded (Lew et al., 1991; Won et al., 1992; Palmero et al., 1993).

With regard to the function of D-type cyclins, increasing evidence points to the notion that they have additional roles other than cell cycle regulation in terminally differentiated cells. For instance, cyclin D1 is known to mediate apoptotic neuronal cell death (Freeman et al., 1994; Kranenburg et al., 1996). It has also been reported that cyclin D1 increases in the process of differentiation induced by NGF treatment in PC12 cells (Yan and Ziff, 1995). Induction of cyclin D3 is also observed during the differentiation process of the myoblasts to myotubes (Kiess et al., 1995). Recently,

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we have demonstrated that D-type cyclins are expressed in terminally differentiated, mitotically inert organs such as the kidney, liver, and brain, suggesting that D-type cyclins have additional roles other than regulation of the G1/S transition in the cell cycle *in vivo* (Sun et al., 1996).

Spermatogenesis is a highly ordered and complex differentiation process whereby mitotically dividing germ cells become mature haploid spermatozoa. It is a unique and excellent model to investigate gene expression during the differentiation process *in vivo*, because morphologically distinct cell types of a certain differentiation stage can be easily identified and highly enriched populations of specific cells could be separated. Thus, taking advantage of the testicular germ cell system, we attempt to determine the expression patterns of D-type cyclins in male germ cells and their possible roles in spermatogenesis.

Materials and Methods

Animals and tissue preparations

F1 (CBA x C57BL) mice obtained from the Animal Breeding Center in Seoul National University were used as the source of all tissues and cell populations. Mice were maintained under the condition of controlled light (06:00 - 20:00) and dark cycles with food and water *ad libitum*. Tissue-specific expression of D-type cyclins was examined in several tissues including the thymus, liver, kidney, and testis derived from adult (more than 6 wk-old) male mice (except for ovary from female mice).

Separation of testicular cells

Enriched populations of testicular cells were obtained by a unit gravity cell separation apparatus previously described by Wolgemuth et al. (1985). Ten sexually mature F1 male mice were sacrificed, testes were obtained and placed into RPMI media at room temperature. The tunica albuginea was removed and the tubules were incubated with 25 ml of 1 mg/ml collagenase for 12 min at 34℃. The tubules were allowed to settle down and the supernatant was removed. Then, the tubules were washed twice with 25 ml of RPMI and incubated with 25 ml of 0.25 mg/ml trypsin and 1 mg/ml DNase I under the same conditions for collagenase incubation. Afterwards, the number of cells was counted and 2.5×10^8 cells were loaded into the gravity unit chamber filled with continuous 1-4% bovine serum albumin gradient. After a 50 min sedimentation, cells were fractionated into 100 fractions. After monitoring purity by morphological examination under the phase-contrast microscope, fractions containing individual cell types were pooled. The purity was approximately 75% for spermatocytes, 80% for round spermatids, and up to 98% for elongated spermatids.

Polysomal RNA separation

Polysomal RNA was fractionated as described elsewhere (Kwon et al., 1993). Briefly, decapsulated adult testes were subjected to homogenization with lysis buffer (15 mM, pH 7.5 Tris-HCl, 60 mM KCl, 15 mM NaCl, 0.3 M sucrose, 0.5% NP-40). The homogenates were centrifuged at 16,000 x g for 30 min. This postmitochondrial supernatant was layered over a continuous 10-40% sucrose gradient in a 14×89 mm polyallomer ultracentrifuge tube (Beckman, USA). EDTA was treated to the fraction at a final concentration of 100 mM and incubated at 30℃ for 10 min before layering over the sucrose gradient. The gradients were centrifuged at 100,000 x g for 4 h at 4℃ in a SW41 rotor (Beckman, USA). After centrifugation, RNA was fractionated into 10 tubes and precipitated with ethanol. These fractions were centrifuged and the RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczinski and Sacchi, 1987). Equal aliquots of purified RNA were used for northern blot analysis.

Northern blot hybridization analysis

The procedure for northern blot hybridization used was as described previously (Sun et al., 1996). Briefly, total RNA was isolated by an AGPC method with minor modifications (Chomczynski and Sacchi, 1987). Total RNA was separated on a 1% denaturing agarose gel and transferred to a nylon membrane (Nytran, Schleicher & Schuell). For the detection of cyclin D1, D3, and CDK4 mRNA, hybridization was performed with the murine cDNA probes (Matsushime et al., 1991). Probes were labeled with ^{32}P-dCTP (NGH-013H, NEN; 3,000 Ci/mmol) by the hexamer primed labeling method (Feinberg and Vogelstein, 1984). The specific activity of the labeled probe was approximately $1\text{-}2 \times 10^9$ cpm/mg DNA. After hybridization, membranes were rehybridized with 18S probe as a control.

In situ hybridization

In situ hybridization was performed based on the method previously reported (Polak et al., 1988) with a minor modification. Pentobarvital-anesthetized mice were perfused through the left ventricle with phosphate-buffered saline (PBS) followed by cold phosphate-buffered formaldehyde solution (4%) for 15 min. Testis tissues were removed and fixed in the same fixative for 4 h. Then, tissues were incubated in PBS containing 20% sucrose at 4℃ overnight. Tissues were frozen and sectioned 10 mm-thick using a cryostat (American optics). Tissue slices were thawed and mounted onto Probe-on™ plus Microscope Slides (Fisher Scientific, USA). They were incubated in 1 U/ml proteinase K in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA at 37℃ for 3 min. After a brief wash with TE (10 mM Tris, 1 mM EDTA, pH 8.0), they were acetylated with 0.0025% acetic anhydride in 0.1 M triethanolamine. Samples

were prehybridized at 37℃ for 2 h in prehybridization solution containing 2x SSC, 250 mM NaCl, 5x Denhardt's solution, 12.5 mM Tris-HCl (pH 8), 12.5 mM EDTA (pH 8.0), and 10% Dextran sulfate. The hybridization mixture was identical to the prehybridization mixture except for the addition of 10 mM dithiothreitol (DTT), 0.5 mg/ml yeast tRNA, and ^{35}S-UTP labeled cyclin D3 riboprobe (5×10^5 cpm). For the preparation of cyclin D3 riboprobe, the full-length cDNA was excised and subcloned into the *Xho*I site of pGEM4Z (Promega) and subjected to *in vitro* transcription according to the manufacturer's instructions (Promega, USA). The hybridization mixture was applied to the tissue sections and incubated in humidified boxes at 65℃ for 24 h. Then, RNase A was treated for 10 min at 37℃ and the slides were washed sequentially with 2x SSC, 1x SSC, 0.5x SSC containing 10mM DTT. The slides were further washed in 0.1x SSC at 65℃ for 30 min, and at RT for 10 min. The sections were then dehydrated by dipping into 50%, 60%, 95%, and 100% ethanol for 3 min. The sections were dipped in Kodak NTB2 emulsion (diluted to 1:1 with water) and stored at 4℃ for 14 d. They were then developed, fixed in a Kodak fixer, stained with Crezil violet, and viewed on a Leitz photomicroscope (Leitz, Germany) under a bright field and dark field. Background labeling was determined from the sections hybridized with an identical quality of radioactive probes in the sense orientation.

Results

Testis-specific expression of cyclin D1 and D3 transcripts

The expression patterns of cyclin D1 and D3 transcripts in various tissues were examined by northern blot analysis (Fig. 1A). As previously reported (Sun et al., 1996), cyclin D1 mRNA (4.2 kb in size) was abundant in the kidney, but not in the thymus, liver, and ovary. Transcripts of cyclin D1 were also detected in the testes at a low level. On the other hand, two different sizes of cyclin D3 transcripts (approximately 2.3 kb and 1.8 kb in size, estimated on 1% denaturing agarose gel electrophoresis) were detected in the adult testis using a full-length cDNA probe. The 1.8 kb transcript expression was particularly testis-specific.

We then attempted to determine the origin of testis-specific cyclin D3 transcripts. At first, to determine whether two transcripts have different lengths of the poly(A) tail, we estimated the sizes of deadenylated transcripts in the testis. After deadenylation, the 2.3 kb transcript was shortened to approximately 1.9 kb in size, which corresponds to the size of the full-length cDNA (Matshushime et al., 1991). Testis-specific cyclin D3 transcript (1.8 kb in size) was also shortened to about 1.4 kb in size (data not shown). Thus, the difference between the two transcripts cannot be attributed to the lengths of the poly(A) tail. Secondly,

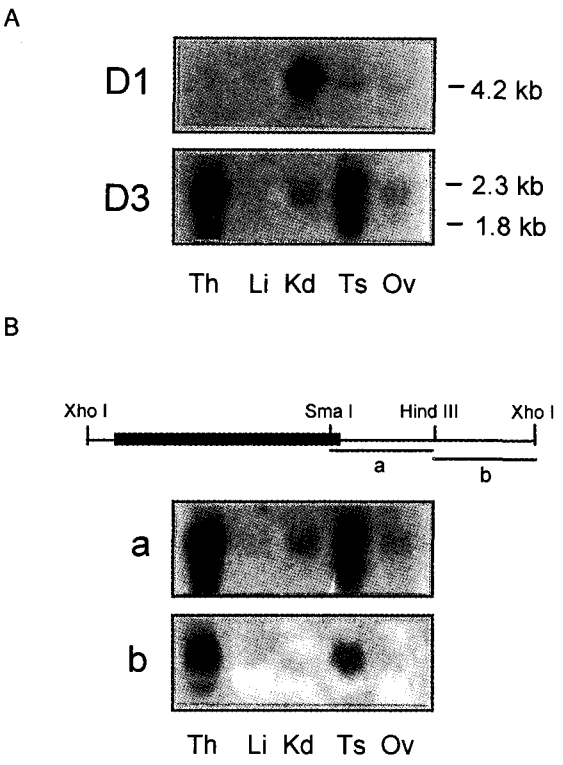

Fig. 1. A. Expression of cyclin D1 and D3 transcripts in the adult testis. Total RNA (30 μg/lane) from various tissues was electrophoresed in a 1.0% denaturing agarose gel, blotted onto a nylon membrane and hybridized with probes of cyclin D1 and D3. After hybridization, membranes were rehybridized with 18S rDNA probe as an internal control (data not shown). Experiments were repeated at least three times and the representative figure is shown. Th, thymus; Li, liver; Kd, kidney; Ts, testis; Ov, ovary. B. Testis-specific cyclin D3 transcript (1.8 kb) originated from the truncation of the 3′-end. Note that a probe (named 'b') derived from the 3′-proximal parts of cyclin D3 cDNA failed to detect the 1.8 kb transcript.

to determine the possibilities of alternative splicing and/or different usage of the 3′-terminal sequence, we used various cDNA probes corresponding to different sequences (Fig. 1B). Among them, only the *Hind* III-*Xho* I fragment of cyclin D3 (0.4 kb in size) designated probe 'b' which hybridized the proximal sequences of the 3′-end, failed to detect 1.8 kb transcripts. Therefore, it appears that two forms of cyclin D3 transcripts could be derived from the same coding sequences.

Developmental stage-dependent activation of cyclin D3 transcript in the testis

In order to investigate the developmental regulation of cyclin D3 in the testis, we analyzed the temporal expression of the cyclin D3 transcript in the developing testes from newborn to adult (more than 6-wk-old) during a one week interval. Since the testis is known to sequentially develop and present a characteristic set of spermatogenic cells at specific ages after birth, we took advantage of this model to determine which types of testicular cell(s) express cyclin D3 transcripts.

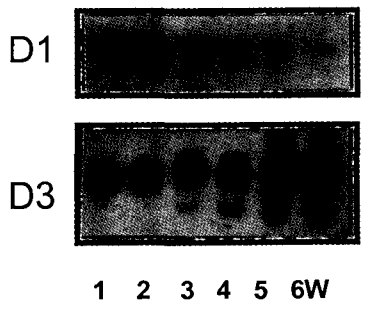

Fig. 2. Developmental stage-specific expression of cyclin D1 and D3 in mouse testes. Testes were obtained from 1 week-old to 6-week old mice at one week intervals. After hybridization with cyclin D3 probe, membranes were rehybridized with cyclin D1. Experiments were repeated three times and the representative figure is shown.

Fig. 2 shows the representative result of northern blot hybridization. Expression of cyclin D3 mRNA was decreased in the 2-wk-old mouse testis and appeared again in the 3-wk-old mouse testis. Interestingly, the 1.8 kb testis-specific cyclin D3 transcript first appeared in the 3-wk-old mouse. Subsequently, both cyclin D3 transcripts were increased and sustained at high levels throughout adulthood. Since postmeiotic spermatid cells first appear around 18 d after birth, it is possible that the expression of the 1.8 kb transcripts may be restricted to the postmeiotic cells. In contrast, the expression of cyclin D1 gradually decreased along with testicular development.

Expression of cyclin D3 transcript is restricted to the meiotic germ cells

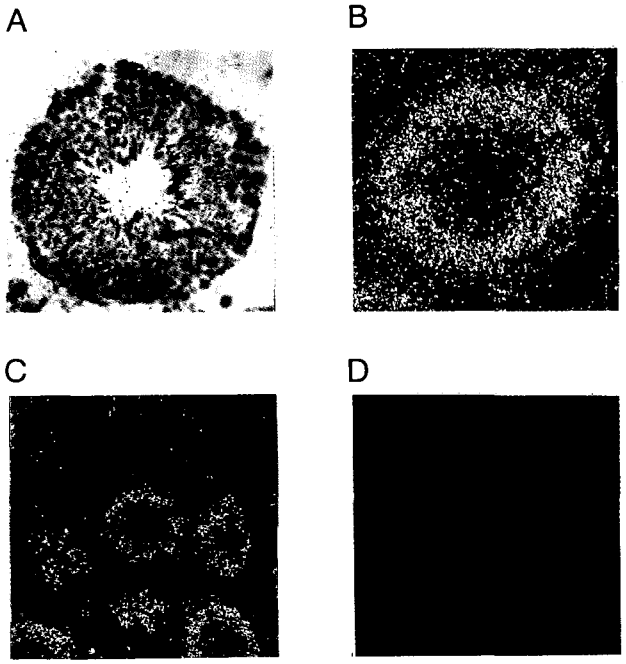

Fig. 3. Localization of cyclin D3 transcripts in the adult testes by *in situ* hybridization. Tissue slices of adult testis were hybridized with ^{35}S-labeled antisense (A-C) or sense (D) riboprobes. Testis sections were photographed using bright-field (A) or dark-field (B-D) optics. Bars=50 mm.

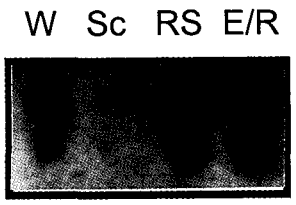

Fig. 4. Expression of cyclin D3 in the postmeiotic male germ cells. Enzymatically dissociated testicular cells were purified by a unit gravity chamber method described in Materials and Methods. Fifteen grams of total RNA from purified cells were electrophoresed and analyzed. W, whole testis; Sc, spermatocytes; RS, round spermatids; E/R, elongated spermatids/residual bodies.

Cyclin D3 transcript raised the possibility that they are abundantly expressed in germ cell lineage. Therefore, to examine the cell type-specific expression pattern of cyclin D3 transcript in detail, we employed *in situ* hybridization techniques (Fig. 3A-D). Hybridization of the antisense RNA probe (full length) gave strong signals in the round spermatid and elongated spermatid layers in a cross-section of the seminiferous tubule. Dark-field images clearly demonstrated that the expression of the cyclin D3 transcript was seminiferous, tubular stage-specific (Fig. 3B-C). On the other hand, no signal was observed when a sense probe was used (Fig. 3D).

To resolve the expression profiles of the two transcripts during germ cell differentiation, we purified the specific cell types from adult testes. As shown in Fig. 4, the 2.3 kb of cyclin D3 transcript was abundantly expressed in the round spermatids, but not in the elongated spermatids and residual bodies. On the other hand, the 1.8 kb testis-specific cyclin D3 transcript was expressed in the round spermatid fractions, and the signal was even stronger than that of the 2.3 kb transcript in the residual bodies/elongated spermatid. Both the 2.3 kb and 1.8 kb transcripts were marginally detected in spermatocytes.

In many cases, transcripts which exist after the spermatid stages are known to be translationally inactive. Thus, we attempted to determine whether cyclin D3 transcripts were translationally active. The sucrose gradient efficiently separated the polysomal

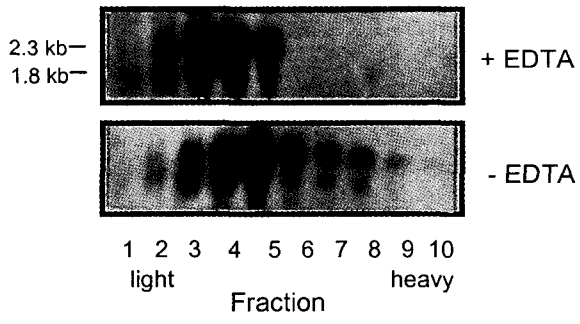

Fig. 5. Distribution of cyclin D3 transcripts in the polysomal RNA separation fractions from mouse testis. Cytosolic fractions were loaded onto 10-40% sucrose gradient and ultracentrifugated at 100,000 g. The same aliquots were electrophoresed and analyzed. Experiments were performed twice and the representative result is shown.

RNA which are translationally active and monosomes which are inactive. Analysis of the northern blot with the cyclin D3 probe indicated that the 2.2 kb transcripts were present in fractions 3-9, while the 1.8 kb testis-specific bands dominated fractions 3-6 (Fig. 5, upper panel). A shift in the distribution of ribosome-associated cyclin D3 mRNA was observed after pretreatment with EDTA. Both two cyclin D3 transcripts were restrictively represented in fractions 3-5 (Fig. 5, lower panel), where dissociated ribosomal RNAs were presented. Therefore, the presence of cyclin D3 transcripts in fractions 6-9 suggests that they are translationally active.

Discussion

In the present study, we clearly demonstrated that cyclin D3 was expressed in the postmeiotic, differentiated spermatids. Although it is premature to estimate the functional importance of postmeiotic cyclin D3, a high level expression of cyclin D3 in postmeiotic spermatids suggests that it may play a functional role in the development and differentiation of spermatozoa. The protein level of cyclin D3 was not determined, but our polysomal RNA separation results indicate that some cyclin D3 transcripts are translationally active. Therefore, it is presumable that the transcript of cyclin D3 in postmeiotic germ cells is translated into the functional protein.

Determining the catalytic partners of testicular cyclin D3 may provide clues to its function. In many somatic cells, D-type cyclins form complexes mainly with cdk4 (Matushime et al., 1992). However, the cdk4, a prime candidate for catalytic partner, may not be a partner for cyclin D3 in germ cells. In fact, cdk4 is down-regulated during testicular development, and the expression of cdk4 is restricted to the mitotic spermatogonial cells and non-proliferating Sertoli cells (Rhee and Wolgemuth, 1995). In respect to cellular localization of the cdk family, Pctaire-1 and Pctaire-3 may be good candidates for the catalytic counterpart of cyclin D3 (Rhee and Wolgemuth, 1995). Pctaires were isolated by homology with cdc2 kinases, and they have serine/threonine kinase activity (Okuda et al., 1992). However, it is largely unknown what their functional roles are and which kinds of cyclins are necessary for the action of Pctaires. Another candidate might be cdk5. Cyclin D3 prefers to interact with cdk5 in some cell types (Zhang et al., 1993). Furthermore, cdk5 is moderately expressed in the testis, although its cellular localization is not yet determined (Tsai et al., 1993). It is of interest to note that cdk5 is known to have an additional role in the brain. Cdk5 phosphorylates the neurofilament proteins, which may participate in the regulation of neurite outgrowth in differentiating neurons (Hisanaga et al., 1993; Shetty et al., 1993). Since re-organization of cytoskeletons precedes morphological and biochemical transformations during spermiogenesis, it is possible to presume that a cdk5-cyclin D3 complex may participate in the re-modeling of the cytoskeleton during spermiogenesis. Equally, it is possible that there are yet unidentified testis-specific kinases in the testis.

One of the interesting findings is the existence of testis-specific cyclin D3 transcript (1.8 kb in size). It is conceivable that this 1.8 kb transcript may result from the utilization of alternative polyadenylation signals of the cyclin D3 gene, although sequence data of the cyclin D3 demonstrated no consensus polyadenylation signal within the 0.6 kb upstream to the 3′-end (Hosokawa et al., 1994). In terms of physiological significance, the shortened cyclin D3 transcript seems likely to be non-functional, since the majority of this transcript is translationally inactive. A large body of evidence suggests that testis is a specialized organ which has distinct transcriptional and/or posttranscriptional regulatory machinery (Davies and Willison, 1993). During the differentiation process, these machineries may turn on and produce several different transcripts as by-products. Thus, the existence of testis-specific transcript of cyclin D3 may infer that the cyclin D3 gene is regulated by the testis-specific transcriptional and/or posttranscriptional machinery.

Wolgemuth and her co-workers (1995) recently reported testicular expression patterns of D-type cyclins, which were very similar to our findings. They found that three different transcripts of cyclin D3 (2.7, 2.3, and 1.8 kb) are expressed in the testis, and the 2.7 and 1.8 kb transcripts were germ-cell specific. Moreover, cyclin D3 transcripts were abundant in the round spermatids as judged by *in situ* hybridization. In the present study, we found that the expression of cyclin D3 is highly restricted in the postmeiotic, haploid spermatids. We resolved the expression patterns of 1.8 kb and 2.3 kb transcripts by cell separation preparation techniques. Furthermore, the 1.8 kb transcript of cyclin D3 may be a result of shortening the 3′-end, and they seem to be translationally inactive while some populations of 2.3 kb transcripts are active as determined by polysomal RNA separation. In spite of many similarities, we were unable to observe the 2.7 kb transcript in the testis. There might be a mouse strain-specificity (F1 (CBA x C57BL) vs. Swiss Webster mice). Our preliminary data showed that ICR mice expressed low amounts of the 1.8 kb transcript without the 2.7 kb transcript in the testis. It seems likely that the different sizes of cyclin D3 transcripts might be by-products of germ cell development, which were functionally inert.

In summary, we demonstrated that cyclin D3 was expressed in postmeiotic male germ cells, suggesting the possible roles, other than cell cycle related function, in the process of differentiation of germ cells.

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