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Expression of a Novel Isoform of Cyclin I in Human Testis

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A new isoform of cyclin I, designated cyclin I_{TI}, was cloned from the human testis cDNA-λgt10 library by using a ~0.5 kb PCR fragment that was obtained by using primers based on the published cyclin I sequence from the human brain. The cyclin I_{TI} cDNA is 1443-bp long and has an ORF of 178 aa with the first ATG Met start codon at nt 1 and has the well-conserved sequence of cyclin box. The 5′ flanking sequence of 728-bp has a high similarity (99.6%) with the 5′-intron of the human β-polymerase gene. The nt sequence of cyclin I_{TI} cDNA has high similarity (99.3%) with the cyclin I gene sequence. The cyclin I_{TI} was found to have three amino acids mutations at nt 25, 172, 223, respectively, including one in the well-conserved sequence of cyclin box. Northern blot analysis indicated that the cyclin I_{TI} is expressed in human testis at increased level compared to other tissues. The in vitro translated protein of the cyclin I_{TI} cDNA was recognized specifically by the antibodies raised against the human sperm proteins, indicating its presence in human sperm. This new isoform may have a physiological role in spermatogenesis and/or human sperm cell function—especially in capacitation and/or acrosome reaction.

Key Words: **cyclin; human testis; sperm; fertility.**

Cyclins are a family of proteins implicated in the induction and control of mitosis. They are characterized by their accumulation in the interphase and dramatic destruction at the end of each cell cycle via the ubiquitin pathway (1,2). Nine classes of cyclins (designated cyclin A through I) have been reported so far in animal cells; several of them have multiple members (3-11). The cyclins have been found to contain a well-conserved amino acid sequence known as the cyclin box (4,12). All proteins designated as cyclins contain this structural motif, which has information necessary for binding to and activation of cyclin dependent kinases (CDKs) (13-15).

Our laboratory investigated the presence and role of the cyclin A (p60) and cyclin B (p62) in human sperm (16). The data indicate the presence and possible involvement of these cyclins in the human sperm cell function, especially capacitation and/or acrosome reaction. The presence and possible role of other cyclins and their interaction have not been examined in the human sperm. Cyclin I has been cloned and sequenced from an equalized cDNA library derived from human forebrain cortex (11). It contains a typical cyclin box near the N-terminus and a PEST-rich sequence near the C-terminus, and shows the highest sequence similarity in the cyclin box to cyclin G and E. Cyclin I mRNA is expressed at high levels in post-mitotic tissues, including skeletal muscle, heart, and brain, and is expressed constantly during cell cycle progression, and thus functions independently of the cell cycle control (11). Its presence and role in human testis and sperm have not been examined.

Based upon the above findings, the present study was conducted to examine the expression of cyclin I in human testis and sperm. The long term objective of the study is to examine interactions among various cyclins and investigate their role, if any, in spermatogenesis and sperm cell function.

MATERIALS AND METHODS

Library screening, and isolation and sequencing of cDNA. The human testis cDNA library in λgt10 was plated at a density of 10×10^3 plaque-forming units (pfu) per 100-mm Petri dish with *Escherichia coli* C600 as host bacterium. The plates were incubated at 37 °C until the plaques reached a diameter not exceeding 1.5 mm. The nitrocellulose filters were placed on the plates for 60 sec, and then peeled off, denatured and neutralized, followed by UV crosslinking. The probe used for screening the library was a fragment generated by the polymerase chain reaction (PCR) the human testis cDNA library in λgt10 by using the primers based on the published cyclin I gene sequence (11). The 5′ primer sequence was 5′-ATGAAGTTTCCAGGGCCT-3′ from position nt 1 to 18 and the 3′ primer sequence was 5′-GATGGGCTCAATTTGGGC-3′ from position nt 534 to 517. The thermoprofile was: 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; 35 cycles. The PCR fragment was separated on 1% agarose gel containing ethidium bromide, and

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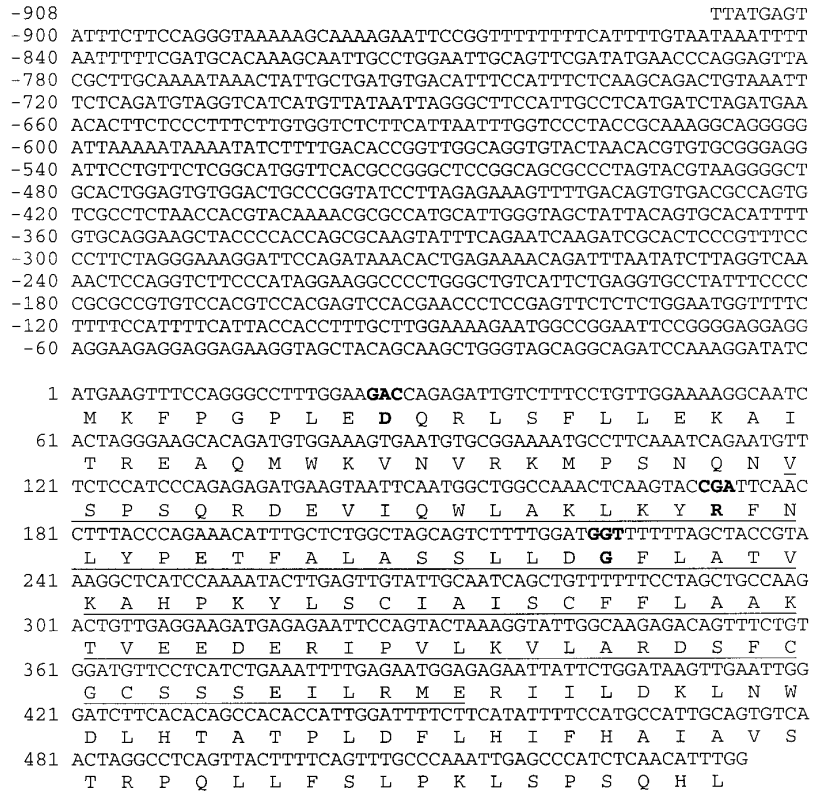

FIG. 1. Nucleotide and deduced amino acid sequences of cyclin I_{TI} cDNA. The human testis cDNA-λgt10 library (Clontech, CA) was screened with a ~0.5 kb PCR fragment based on the published human cyclin I sequence [11]. The ~1.4 kb insert of the positive clone was amplified by the λgt10 insert screening amplimers, then subcloned into pBluescript II SK+ at EcoR1 sites (Stratagene, La Jolla, CA), and sequenced. The sequencing thermoprofile was: 94°C for 1 min, 50°C for 2 min, 72°C for 3 min; 35 cycles. The underline indicates the cyclin I box.

the desired band was eluted, extracted with phenol/chloroform, and precipitated with ethanol. It was labeled by random hexamer method (Life Technology, GIBCO, BRL).

For screening the library, the filters were prehybridized in QuickHyb solution (Strategene, CA) for 30 min at 65°C, and then incubated with the ^{32}P-labeled probe for 3 hr at 65°C. The filters were washed (×3) in 2xSSC containing 0.1% SDS, and then twice in 2xSSC buffer at room temperature. The filters were wrapped in plastic sheet and exposed to a x-ray film with an intensifying screen for 2 days at −70°C. The putative positive clones were picked, and subjected to second and third screening. The phage DNA of the positive clones was purified, and subjected to EcoRI digestion, then separated on 1% agarose gel containing ethidium bromide. The insert was eluted from the gel, purified, subcloned into pBluescript II SK + at EcoRI site and then sequenced by using T3 and T7 promoter primers, and the internal primers based on the obtained sequences with the dsDNA Cycle Sequencing System (Life Technologies, GIBCO, BRL) (18,19). The sequencing thermoprofile was the same as described above.

The search for nt and aa sequence homology in the GenBank, National Biomedical Research Foundation (NBRF), and Swiss sequence banks was performed using Fasta (Genetic Computer Group, GCG, compiled by the University of Wisconson, at Madison, WI) (20).

Northern blot procedure. The human testis cDNA library in λgt10 and the human endocrine system multiple tissue northern (MTN) blot were from Clontech, CA. The MTN blot was prehybridized (15 min, 65°C) with QuickHyb solution (Strategene, CA) and then probed (2 hr, 65°C) with ^{32}P-labeled insert cDNA. The probe was prepared by eluting the cDNA from the pBluescript vector by EcoRI digestion, and was labeled by the random hexamer method [18]. The membrane was washed, and exposed to x-ray film as described above. After the membrane was stripped of the cDNA probe, it was rehybridized (2 hr, 65°C) with ^{32}P-labeled β-actin (Clontech, CA) probe, washed, and exposed as above.

In vitro transcription and translation procedure. The recombinant clone was digested with Xhol or Notl to linearize the plasmids for run-off transcription using T7 and T3 RNA polymerase (Promega, Madison, WI) (21). The post-restriction-digested DNA was incubated (30 min, 37°C) with proteinase K, followed by phenol/chloroform extraction, and ethanol precipitation. One μg of the restricted, proteinase K-treated DNA template, was incubated (30 min, 37°C) with 5 μl of 5x transcription buffer containing 2 μl of 100 mM DTT, 20 units of RNasin ribonuclease inhibitor, 1 μl of 10 mM dNTP, and 10 U of T3 or T7 RNA polymerase in a volume of 20 μl. Template DNA from the transcription reaction was digested (15 min, 37°C) with RNase-free DNase (1 U/μg template DNA), followed by phenol/chloroform extraction and ethanol precipitation. The transcribed RNA was dissolved in diethylpyrocarbonate-treated water and used for the in vitro translation procedure (Promega, Madison, WI). The template mRNA was heated for 10 min at 67°C and immediately cooled on ice. mRNA (100 ng/μl, final concentration) were translated (60 min, 30°C) in a total volume of 50 μl reaction mixture containing 35 μl of rabbit reticulocyte lysate-nuclease treated, 1 μl of 1 mM amino acid mixture minus methionine, 1 μl of RNasin ribonuclease inhibitor (40 units/μl), and 4 μl of ^{35}S-methionine (10 mCi/ml). Luciferase RNA was used as a control. After the reaction was complete, a 5 μl aliquot was analyzed by using the non-reducing condition in the slab SDS-PAGE (5-10%), followed by autoradiography as described elsewhere (21).

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cyclin I    M K F P G P L E N Q R L S F L L E K A I 20
cyclin I_TI . . . . . . . . D . . . . . . . . . .

cyclin I    T R E A Q M W K V N V R K M P S N Q N V 40
cyclin I_TI . . . . . . . . . . . . . . . . . . .

cyclin I    S P S Q R D E V I Q W L A K L K Y Q F N 60
cyclin I_TI . . . . . . . . . . . . . . . . . R . .

cyclin I    L Y P E T F A L A S S L L D R F L A T V 80
cyclin I_TI . . . . . . . . . . . . . . G . . . . .

cyclin I    K A H P K Y L S C I A I S C F F L A A K 100
cyclin I_TI . . . . . . . . . . . . . . . . . . .

cyclin I    T V E E D E R I P V L K V L A R D S F C 120
cyclin I_TI . . . . . . . . . . . . . . . . . . .

cyclin I    G C S S S E I L R M E R I I L D K L N W 140
cyclin I_TI . . . . . . . . . . . . . . . . . . .

cyclin I    D L H T A T P L D F L H I F H A I A V S 160
cyclin I_TI . . . . . . . . . . . . . . . . . . .

cyclin I    T R P Q L L F S L P K L S P S Q H L     178
cyclin I_TI . . . . . . . . . . . . . . . . .
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FIG. 2. Comparison of amino acid sequences between the cyclin I_{TI} isolated from human testis and the cyclin I isolated from human brain. The dots indicate identical amino acids.

Western blot procedure. The in vitro translated products were analyzed by using the SDS-PAGE and Western blot procedure (18-19). Briefly, 20 μl of the in vitro translated product(s) were run under non-reducing condition in the slab SDS-PAGE (5-10%), electrophoretically transferred from the gel to nitrocellulose membrane, and the Western blot strips were incubated with the antisperm Ab/pre-immune Ig/control Ig ($\sim$1 μg/ml), and the reacted bands were visualized as described elsewhere (18). The antisperm antibody was raised in rabbits against the human sperm proteins solubilized in 0.3 M lithium diiodosalicylate (LIS) as described elsewhere (17). The antibodies were purified on a protein A immunocolumn and will be referred to as "antisperm Ab" (17). The antisperm Ab binds predominantly to the acrosomal region of the human sperm cell and inhibits sperm cell function in various functional assays (17). The immunoglobulins isolated from serum of control animals injected with buffer without any protein will be referred to as "control Ig." The control Ig or the pre-immune Ig was used as a control in various experiments. The control Ig and the pre-immune Ig do not bind to human sperm cell and have no effect on its function.

RESULTS AND DISCUSSION

Upon screening 4×10^6 pfu from the library with the $\sim$ 0.5 kb PCR fragment, three positive clones were found. One clone that has the largest insert of $\sim$ 1.4 kb, was selected for further analysis. The nucleotide (nt) sequence was established by total sequencing of both strands by using T3 and T7 promoters and four internal primers, and each segment was sequenced several times. The alignment of sequences yielded a 1443-bp cDNA (Fig.1). Computer translation (GCG program, Madison, WI) of the cDNA generated an open reading frame (ORF) of 178 amino acids (aa). The deduced aa sequence is shown below the cDNA sequence in Figure 1.

Extensive computer search in the database indicated that the cDNA clone had a unique sequence and has the well-conserved cyclin box sequence. A 728-bp segment of the cDNA from nt position -860 to -132 has a high similarity (99.6%, Besfit) with the human β-polymerase gene (accession number J04201) at its 5′ intron (nt position 324 to 1050). A 535-bp segment of the cDNA from nt position 1 to 535 has a high similarity (99.3%, Besfit) with the human cyclin I gene (accession number D50310) from nt position 1 to 535 (Fig.2). A 132-bp segment between these two regions having high similarities showed a novel sequence.

The high similarity with the human cyclin I sequence, especially in the conserved cyclin box, indicated that the cDNA is a member of the cyclin family and is related to cyclin I. However, there were four nucleotides and three amino acids that were different from the cyclin I sequence (Fig. 2). At position 25, A changed to G, transversion at the first codon, resulting in 9th amino acid N changed to D. At position 172, C changed to G, transversion at the second codon, resulting in the 28th amino acid Q changed to R. At position 225, A changed to G, and at position 227, G changed to T, transversion at the first and third codons, respectively, resulting in 35th amino acid R changed to G. Although, the 535-bp segment of the cDNA only has a partial sequence of the human cyclin I, it contains the complete cyclin box (underlined amino acids in Fig.1). The cyclin box has a well-conserved amino acid sequence (4,22). Based upon these findings, the cDNA

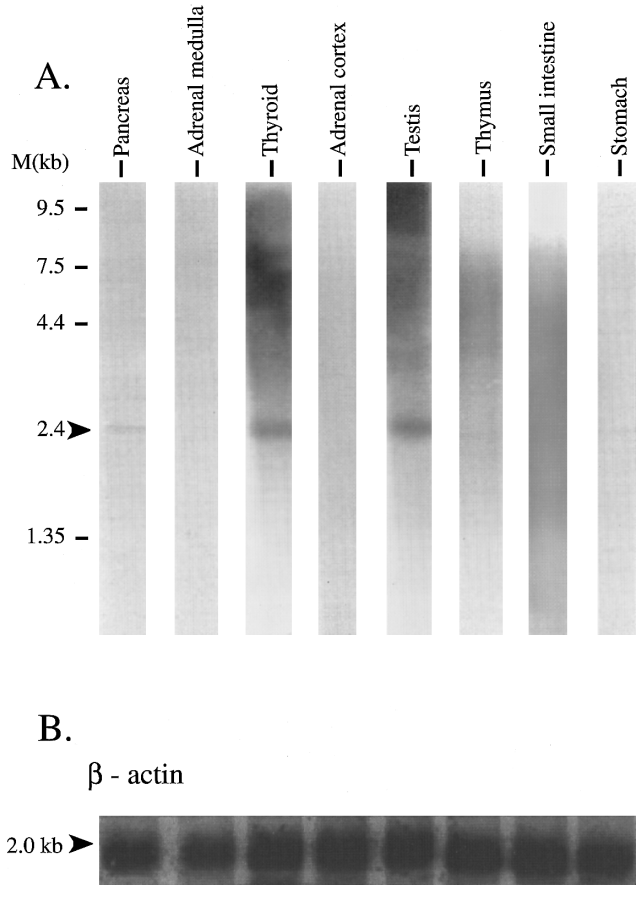

FIG. 3. Human multiple tissue Northern blot probed with the cyclin I_{TI} cDNA (Panel A). The blot contains approximately 2 μg of poly $(A)^+$ RNA per lane from eight human tissues. The blot was prehybridized with QuickHyb solution (Strategene, CA), incubated (2 hr, 65°C) with the ^{32}P-labeled cyclin I_{TI} cDNA probe, washed and exposed to x-ray film for 2 days to 2 weeks. The probe was prepared by the random hexamer method (GIBCO, BRL). After the blot was stripped off the cyclin I_{TI} cDNA probe, it was hybridized (2 hr, 65°C) with the ^{32}P-labeled β-actin probe (16), washed, and exposed as above (Panel B).

seems to be an isoform of cyclin I present in the human testis, and thus, was designated as cyclin I - testis isoform (cyclin I_{TI}). Although cyclin I is broadly distributed among various tissues, different tissues may have different isoforms.

The human multiple northern blot contains approximately 2 μg of poly $(A)^+$ RNA per lane from eight different human tissues, namely pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine and stomach (Clontech, CA). The blot was hybridized with the cyclin I_{TI} cDNA probe released from pBluescript II SK+ vector by EcoRI digestion. When the hybridization was conducted at 65°C, one major band of $\sim$ 2.4 kb in thyroid and testis lanes was apparent (Fig.3, Panel A). However, when the hybridization was conducted at lower stringency (56°C) and the blot was exposed for a longer time (up to 2 weeks), the other tissue lanes also showed a weaker band at $\sim$ 2.4 kb (data not shown). However, the $\sim$ 2.4 kb band in the thyroid and testis lanes were still the most prominent. After the blot was stripped off the cyclin I_{TI} cDNA probe and rehybridized with the ^{32}P-labeled β-actin probe, all lanes showed a positive signal of equal intensity, indicating integrity and equal loading of mRNA from each tissue (Fig.3, Panel B).

Cyclin I is thought to be broadly expressed in the human tissues because of its possible role in the cell cycle progression (11). However, its highest expression was observed in heart and skeletal muscle, which contain only a few dividing cells. Liver had no expression, and the placenta showed very low level expression, however, it was the most rapidly dividing tissue among the eight tissues examined. These findings indicate that the cyclin I expression may have some additional role, besides its direct or indirect involvement in the cell cycle control. In our studies the highest level of cyclin I_{TI} was observed in the testis. The testis is the site of spermatogenesis that involves active cell division, proliferation and differentiation. It is possible that each isoform of cyclin I is associated with some aspect of the cell cycle progression in a cell/tissue-specific manner.

The cDNA was cut from λgt10, and subcloned into pBluescript II SK+ vector. Double-stranded DNA sequencing of the recombinant plasmid established the orientation of the mRNA-like strand sequence, with reference to either the T3 or T7 RNA polymerase promoter sequences flanking the insert sequence. Digestion of the recombinant plasmid with Xhol produced a fragment containing the vector sequence at one end and the cDNA sequence at the other end of the T3 promoter. Notl digestion of the recombinant plasmid yielded a fragment with T7 promoter sequence flanked with the cDNA sequence on one side and the vector sequence on other side. Run-off transcripts generated by the T7 and T3 RNA polymerases were translated using the rabbit reticulocyte lysate system. The products were resolved in the SDS-PAGE, and then the gel was either subjected to autoradiography or electrophoretic transfer to nitrocellulose membrane for the Western blot analysis.

Autoradiography of the gel showed a single band of $\sim$ 20 kD in the in vitro translated product of the T7 RNA polymerase, using the Notl-digested recombinant plasmid, indicating it to be sense strand (Fig. 4). Run-off transcripts generated by T3 RNA polymerase using the Xhol-digested recombinant plasmid, did not yield any translated product, indicating it to be antisense strand. Computer generated amino acid sequence indicated molecular weight of the cyclin I_{TI} protein as 20.388 kD, which is in agreement with the in vitro translated protein ($\sim$ 20 kD). The Western blot analysis showed that the $\sim$ 20 kD band of the in vitro translated product of the sense strand, that was not tran-

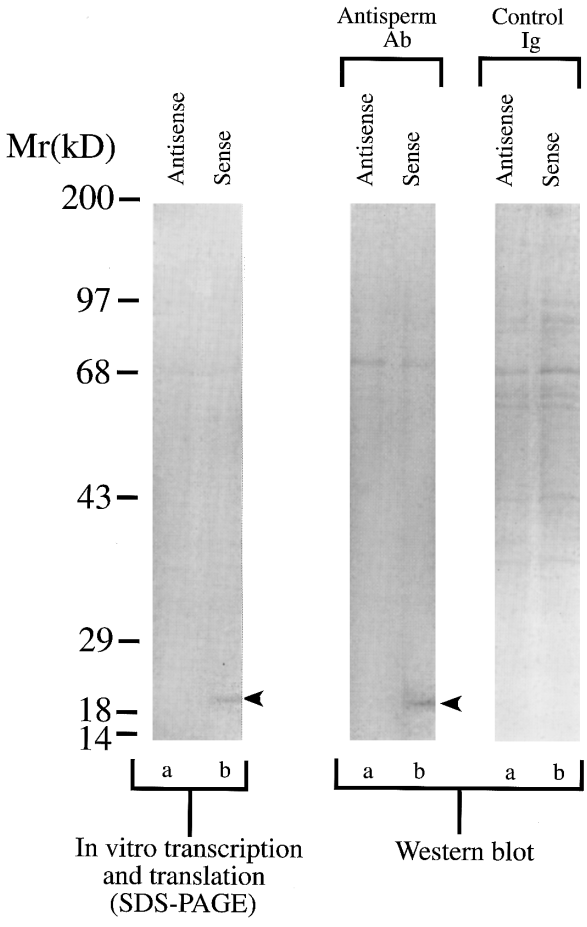

FIG. 4. Analysis of the in vitro translation products. Autoradiograph of the dried SDS-PAGE gel (5-10%) of the in vitro translation products of various run-off transcripts. Translation product of the NotI-digested recombinant plasmid T7 transcript is shown in lane b. Translation product of the XhoI-digested recombinant plasmid T3 transcript is shown in lane a. Western blot analysis of the in vitro translation product of the NotI-digested or XhoI-digested recombinant plasmid T7 transcript probed with the antisperm Ab or with the pre-immune/control Ig. Arrow indicates that the in vitro translated protein (~20 kD) was recognized by the antisperm Ab and not by the pre-immune/control Ig.

scribed and translated by the antisense strand, was recognized specifically by the antisperm Ab, and not by the pre-immune/control Ig (Fig.4). The recombinant protein was recognized specifically by the antisperm Ab raised against human sperm proteins, and not by the pre-immune/control Ig, indicating the presence of cyclin I_{TI} in human sperm. Previously, our laboratory demonstrated the presence and role of cyclin A and B in human sperm function (16). The present study indicates the expression of a novel isoform of cyclin I, designated cyclin I_{TI}, in human testis and sperm. The exact function of cyclin I_{TI} in the regulation of spermatogenesis and sperm function, especially capacitation and acrosomal exocytosis requires further study.

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