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Differential Expression of Cyclins B1 and B2 during Medaka (*Oryzias latipes*) Spermatogenesis

Koichi Mita, Takayuki Ohbayashi, Kenichi Tomita, Yohei Shimizu,
Tomoko Kondo and Masakane Yamashita*

*Laboratory of Molecular and Cellular Interactions, Division of Biological Sciences,
Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan*

ABSTRACT—The Cdc2-cyclin B complex (named the M-phase-promoting factor, MPF) is well known to be a key regulator of G2-M transition in both mitosis and meiosis. However, MPF may have functions other than the cell cycle regulation, since its activity is detectable in post-mitotic (or post-meiotic) non-dividing cells. Cyclin B comprises several subtypes, but their functional differences are still unknown. Despite the established function of MPF during oocyte maturation, its role during spermatogenesis, where spermatogenic cells undergo drastic morphological changes after meiosis, remains to be elucidated. To address these issues, we have isolated cDNA clones encoding cyclins B1 and B2 from medaka testis and raised polyclonal antibodies against their products. Using these as probes, we examined the expression patterns of cyclins B1 and B2 in medaka testis at both mRNA and protein levels. Cyclin B1 and B2 mRNAs were expressed in all stages of spermatogenic cells except for spermatozoa, although the expression levels varied according to the spermatogenic stages. Cyclin B1 protein was expressed only in spermatogonia and spermatocytes at prophase and metaphase with a transient disappearance at anaphase. On the other hand, cyclin B2 protein was continuously expressed throughout spermatogenesis, even in spermatogonia and spermatocytes at anaphase and in post-meiotic spermatids and spermatozoa. The difference in their expression patterns suggests that cyclins B1 and B2 have distinct roles in medaka spermatogenesis; i.e., cyclin B1 controls the meiotic cell cycle, whereas cyclin B2 is involved in process(es) other than meiosis.

INTRODUCTION

The cell cycle is controlled by cyclin-dependent kinases (Cdks), which require the binding of regulatory subunits, cyclins, for activation. To date, 8 types of Cdks (Cdks 1 to 8) and cyclins (cyclins A to H) have been designated (Gao and Zelenka, 1997). Each Cdk forms a complex with a definite cyclin and regulates a certain phase of the cell cycle (Nigg, 1993), except the complexes Cdk5/cyclin D, Cdk7/cyclin H and Cdk8/cyclin C, the functions of which are not restricted to the cell cycle regulation. For example, cyclins A and B (called mitotic-cyclins) are regulatory subunits of Cdks 1 and 2 involved in mitosis, while cyclins D and E (called G1-cyclins) regulate Cdks 2, 4 and 6 that function at the G1 or S phase. The partner of Cdc2 (Cdk1) necessary for G2-M transition is B-type cyclins, and the Cdc2/cyclin B complex is known as the M-phase-promoting factor (or the maturation-promoting factor, MPF) (Lohka *et al.*, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Draetta *et al.*, 1989; Labbé *et al.*, 1989; Gautier *et al.*, 1990; Yamashita *et al.*, 1992).

A hallmark sequence found in all cyclins is known as the

cyclin box, which consists of 150–200 amino acids and is required for its binding to Cdks (Kobayashi *et al.*, 1992). B-type cyclins also contain two regions highly conserved in many species. One region known as the destruction box is involved in the destruction of cyclin itself at anaphase by ubiquitin-mediated proteolysis (Peters, 1998; Tokumoto, 1999). The destruction box exists at the amino terminal of cyclin B, consisting of 9 amino acids, RXALGXIXN (where X indicates any amino acid), followed by a lysine-rich stretch (Glotzer *et al.*, 1991). The other conserved region is the FLRRXSK motif found within the cyclin box (Minshull *et al.*, 1989). Although only one type of cyclin B (cyclin B1) has been discovered in goldfish (Hirai *et al.*, 1992), zebrafish (Kondo *et al.*, 1997) and the rat (Markiewicz *et al.*, 1994) to date, many species have several subtypes of cyclin B as is the case for other cyclins; i.e., the human (Jackman *et al.*, 1995), mouse (Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993) and Japanese brown frog *Rana japonica* (Ihara *et al.*, 1998) have cyclins B1 and B2, the domestic fowl has cyclins B2 and B3 (Gallant and Nigg, 1992; Gallant and Nigg, 1994), and the African clawed frog *Xenopus laevis* has cyclins B1 to B5 (Brandeis *et al.*, 1998; Minshull *et al.*, 1989). Subtype-dependent differences in the expression pattern, subcellular localization and phenotype of the ectopic expression or knockout of the genes

* Corresponding author: Tel. +81-11-706-4454;
FAX. +81-11-706-4456.
E-mail. myama@sci.hokudai.ac.jp

were reported for *Xenopus* oocytes (Kobayashi *et al.*, 1991; Stewart *et al.*, 1994; Yoshitome *et al.*, 1998), mouse spermatogenic cells (Brandeis *et al.*, 1998; Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993; Chapman and Wolgemuth, 1994) and human cultured cells (HeLa cells) (Jackman *et al.*, 1995), but their functional differences are still unknown.

The function of MPF in oocyte maturation has been well studied, and its key role in regulating meiosis, as well as mitosis, is fully established (Yamashita *et al.*, 2000). Like oocyte maturation, spermatogenesis comprises meiosis. It is therefore reasonable to assume that MPF also plays an important role in this process. To date, however, no detailed studies have been conducted on the role of MPF during spermatogenesis. In addition to regulation of the cell cycle, MPF is also thought to have another function, as its activity is observed in post-mitotic (and post-meiotic) non-dividing cells, including spermatids in the rat (Gromoll *et al.*, 1997) and newt (M. Ito, M. Yamashita, Y. Matsuda, T. Yamamoto and S.-I. Abe, unpublished). Spermatogenesis comprises the mitosis, meiosis and post meiotic metamorphosis of spermatids (spermiogenesis), providing an excellent experimental system for comprehensive investigation of the MPF function.

To investigate the role of MPF during spermatogenesis, we selected the testis of the teleost medaka *Oryzias latipes* as an experimental system for the following reasons: 1) Under artificial reproductive conditions, medaka undergoes continuous spermatogenesis and oogenesis, providing experimental materials all the year round (Iwamatsu, 1997). 2) Medaka testis consists of cysts, in which spermatogenesis proceeds synchronously (Grier, 1976). This characteristic allows us to identify the spermatogenic stage easily. 3) Medaka spermatogenesis can be reproduced in a cell culture system (Shimizu *et al.*, 1997), enabling us to manipulate genes by microinjection of mRNAs or antisense RNAs into spermatogenic cells.

As the first step towards the understanding of the role of MPF during spermatogenesis, we isolated cDNA clones encoding medaka cyclins B1 and B2, produced antibodies against recombinant proteins, and examined expression patterns of cyclins B1 and B2 in medaka testis at both mRNA and protein levels. The observed difference between the expression patterns of cyclins B1 and B2 imply that they have distinct roles in spermatogenesis.

MATERIALS AND METHODS

Testis extraction

Medaka were purchased from a local fish farm (Yatomi, Aichi Prefecture) and kept in fresh water at 27°C under artificial light conditions (14 hr light and 10 hr dark). Testes were surgically isolated from adult individuals, and the proteins were extracted by extraction buffer (EB) containing higher concentrations of EGTA and HEPES (Iwamatsu *et al.*, 1999a, b) than those used for other species (Tanaka and Yamashita, 1995). The composition of EB was 100 mM β -glycerophosphate, 65 mM $MgCl_2$, 30 mM EGTA, 40 mM HEPES, 1 mM dithiothreitol, 100 μ M (p-amidinophenyl) methanesulfonyl fluoride and 3 μ g/ml leupeptin (pH 7.5). The testes were washed 3 times with ice-

cold EB before extraction, and after removing excess EB with filter paper, 2.5 μ l of new EB per one testis was added. The testes were homogenized with a pestle (Pellet Pestle, Kontes, Vineland, NJ, USA) and centrifuged at 15,000 g for 10 min at 4°C. The supernatant was used for immunoblotting analysis.

cDNA cloning

Fragments of cDNAs encoding medaka cyclins B1 and B2 were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from medaka ovary total RNA isolated with ISOGEN (Nippon Gene, Tokyo, Japan) using two degenerate oligonucleotides corresponding to the highly conserved amino acid sequences in cyclin B, LCQAFSD and KYEEMPPE (Kondo *et al.*, 1997). The RT-PCR products were classified into those homologous to cyclin B1 and to cyclin B2. The putative cDNA clones for cyclins B1 and B2 thus obtained by RT-PCR were then used as probes to isolate full length cDNA clones from a cDNA library constructed from medaka testis (generously provided by Dr. T. Kobayashi, NIBB, Japan).

In situ hybridization

Medaka testes were fixed for 1 hr at 4°C in 0.4 $\times$ PBS (1 $\times$ PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4 , 2.68 mM KCl, 1.47 mM KH_2PO_4 , pH 7.4) containing 2% paraformaldehyde, 2.5% glutaraldehyde and 8% picric acid saturated in 95% ethanol. The fixed testes were washed in 70% ethanol for 10 min, 90% ethanol for 10 min, 99% ethanol 3 times each for 10 min and xylene 3 times each for 10 min. The testes were embedded in paraffin and cut into 2- μ m sections. Hybridization with digoxigenin (DIG)-labeled RNA probes was performed according to Boehringer Mannheim's protocol (Tokyo, Japan), with the exception that 66% formamide was used.

Production of recombinant proteins and antibodies

A recombinant protein containing the full-length of medaka cyclin B1 (T7- Δ 0-cyclin B1) was produced as follows. A cDNA encoding the full open reading frame of medaka cyclin B1 was amplified by PCR with two oligonucleotide primers, 5'-CGGGATCCATGGCTCTGAGAGTTACC-3' and 5'-GTGCTCGAGAGAAATCTGCGTTGCCAG-3'. The PCR product was ligated into the *Bam*HI/*Xho*I site of pET-21a expression vector and expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen, Madison, WI, USA) for the use as an antigen to produce antibodies.

Production of a recombinant protein including the full-length of medaka cyclin B2 was unsuccessful probably because of the instability of the recombinant protein in bacteria. We therefore produced a recombinant medaka cyclin B2 lacking 25 amino acids from its N-terminus (Δ 25-cyclin B2) with the oligonucleotide primers 5'-CGGGATCCGATCCCAGGAGAGCTGCT-3' and 5'-GTGCTCGAGGGGATCGTGACAGAGCATC-3'. The PCR product was ligated into the *Bam*HI/*Xho*I site of pGEX-KG expression vector (Guan and Dixon, 1991) to produce a fusion protein with glutathione S-transferase (GST- Δ 25-cyclin B2) used for an antigen. The same PCR product was also ligated into pET-21a to produce Δ 25-cyclin B2 protein without GST (T7- Δ 25-cyclin B2) for confirming the specificity of antibodies.

T7- Δ 0-cyclin B1 and GST- Δ 25-cyclin B2 expressed in *E. coli* were purified by SDS-PAGE, followed by electroelution in Tris-glycine buffer without SDS, according to the method described previously (Hirai *et al.*, 1992). The purified proteins were dialyzed against 1 mM HEPES (pH 7.0), lyophilized, and injected into mice to produce antibodies as described previously (Yamashita *et al.*, 1991). Polyclonal antibodies against the recombinant cyclins were affinity-purified with the antigenic proteins electroblotted onto an Immobilon membrane (Millipore, Tokyo, Japan).

Immunocytochemistry

The ordinary immunocytochemical techniques were unable to detect cyclin B proteins in the medaka testis probably because of the low protein content in the testis (less than 1 μ g/ml according to calcu-

lation using T7-Δ0-cyclin B1 and GST-Δ25-cyclin B2 as standards, data not shown). We therefore used the enhanced technique with a microwave oven as described by Shi *et al.* (1991). Medaka testes

were fixed in Bouin's fixative for 3 hr at room temperature, dehydrated, embedded in paraffin, sliced at 3 μm in thickness, and placed on slides treated with VECTABOND (Vector Lab., Burlingame, CA, USA).

Cyclin B1

ATGGCTCTGAGAGTTACCAGGAACCGACTGGCCCTCCACCGGGCCGAACATGGGCGGAAAGACGTGCTCGGTGGCCGGGCTACACAGAAG	AAGGC	5
M A L R V T R N R L A S T R A E L G G K T C S V A G P T Q K		95
CCCCGCGGGCCCTCGGAGAAATCGGAAACGTGCGAGTTATTAACAAAGATGTACGAAAAAGACTATCAAGACGGAGGTGCTAAGAAG		185
P R A A L G E I G N V A V I N K D V T K K T I K T E V A K K		60
ACAAAGATCCCAGCTAAAGCTGAGAAGATCGAGCTACCAAAAGCTGTGTGGTCCCTGTTAAACACGACCTGAAGTGCAGGTGCTAG		275
T K I P A K A E K I E L P K A A V V P V K P A P E V Q V T E		90
GTGCCAGACAGGCTGAACCTGCATCCCCACCCCATGGAGACCTCTGGATGTGAGTCTGCTGACCTCTGTCAAGCATTTCAGATGTC		365
V P D Q A E P A S P T P M E T S G C E S A D L C Q A F S D V		120
ATCCTCAACACGGCCATCAGGGACGTGGATGCAGATGACTACGACAACCCCATGCTCTGCAGTGAATATGTTAAGGACATCTACAAGTAC		455
I L N T A I R D V D A D D Y D N P M L C S E Y V K D I Y K		150
CTCCGTGAGTGTGAGATGGAGCAGAGCGTAAAGCCAACTACCTTGAGGGTCAGGAGATCACCGAAACATCGAGCCATCCTCATGTAC		545
L R Q L E M E Q S V K P N Y L E G Q E I T G N M R A I L I D		180
TGGTCTGGTCAAGTGGGCTCAAGTTCGGTCTGCTGCAGGAGCATGTACATGACTGTGGGAATAATCGACCGCTTCTTTCAGGCCAT		635
W L V Q V G L K F R L L Q E T M Y M T V G I I D R F L Q D H		210
CCAGTCCCCAAGAAGCAGCTGCAGCTGGTGGCGTGAACCCCATGTTCTTGGCTCCAAATACGAGGAGATGTACCTCCGGAGATCTCA		725
P V P L K Q L Q L V G V T A M F L A S K Y E E M Y P P E I S		240
GACTTTGCTACGTGACGACAGGCTTACACCACTGCTCAGATCAGAGACATGGAATGACTATTCTGCGAGTGTGAAGTTTCAGCTG		815
D F A Y V T D R A Y T T A Q I R D M E M T I L R V L K F Q L		270
GGCGCGCCCTCCCGCTGCACTTCTCAGAAGGGCTCCAAGATCTATGAGGTAACGTGTAACAACACACCCCTGGCCAAATACCTGCTG		905
G R P L P L Q <u>F L R R A S K</u> I Y E V T A E Q H T L A K Y L D		300
GAGCTCTCCATGGTGTACTACGACATGGCTATTCTCGCCCTCCTTGGTGGCAGTGCAGCTCTGGCGTGACGCTCAAGGTCCTGGAT		995
E L S M V D Y D M A H F S P S L V A S A A L A L T L K V L D		330
GCAGGGGAATGGATGTGACACTGCAGCATTACATGGAGTACACAGCAGAGCCCTGACACCTGTTATGGCGACATCGCTAAGAATGTG		1085
A G E W D V T L Q H Y M E Y T A E T L T P V M A H I A K N V		360
GTGAAGTCAACATGGGCAAACTAAACACATGGCCATCAAAAGGAAATACTCTACATCAAGCAGATGAGGATCGCCACCATCTCTCAG		1175
V K N N G Q T K H M A I K G K Y S T S K Q M R I A T I S Q		390
CTCAATCTCTGTGTGAAGATCTGGCAACGAGATTTCTTAGTGAGGACAGCAGTGTCTCCCGTGTACAGATGGACTGTGTGCTG		1265
L K S S V V K D L A T Q I S *		404
GGATTCTTGGGATTGGGCTTTTACCGACTACAGTTTGTGTTTTTATTTTTTAAAGTGTGCTGAAGTTGTGACAGACAGCCCTC		1355
AATCAGAGTATTTTACCAAAACATCCAACCTCTTGGAGCTCAGTCGGCTACATCTGACCCAGAAATGCACAACTTGTTTTATCTC		1445
TATCATTGTGTAGTAGCCGGCTGAACCTCTGACCCAGCAGGCAATGTTTCTTAAACATGTGGATTTACAACAATATGCTACCTGTGTG		1535
AGGTGACTATGTAATATGTACAGTATTCTTTTAAATTTTGTGTATGGAGTGTCTGATTTTATTTTTTAAATGTATATTAAT		1625
GGCTTATCTAAGATTAAAAA		1662

Cyclin B2

GCAAATGCTTCAGTGTGACTTGTGTGGGCGGTCTACCGACACCGCGGGGATCCCGGCCAGAGTTTGTCTCGTAGTTTCGCGCCGG	GAGGAA	6
ATGTCTCTGTGGAATTTGTGCGCAGCAGCTCTGCGCCGACAGCAGCCCTCGGAGGATGGGGAAGAGCTGCAGCGGATCCCGAGGAGA		96
M S S V E I V A L L A A E H P R R M G K G A A A D P R		186
GCTGCTTTGGGAGAACTGACCACTTGAACGACAGTGCAGCCACCAAGGAAAGTGGGACGACAAAAAACCTTGAAGGCCTCTCTGT		276
A C A L G E L T N L N A A A T N G K V G P A K K P L K A S C		60
GCTCAGAAACCCAACTTACTCAGTTGGTGGCTTCCATGATCCAGACGGGTGCGGCTGCCTCAGCTCCCGTCTTAGCTAAACCCCGCTG		366
A Q K P K L T Q L V A S M I Q T G A A A S A P V L A K P S V		90
AAGGAGGAGCAGGAGCTCTGCCAGGCTTTCTCGGAGGTGCTGCTGCTGTGACGAGCTGGACGAGCAGGATGCAGACAGCCCTCAGTTA		456
K E E Q E L C Q A F S E V L L A V Q D V D E Q D A D Q P Q L		120
TGCTCCCAATATGTCAAGACATCTACAAGTATCTGCACATCCTGGAGGAGCAGCAGCTGTGCGTCCCACTACATGCAAGGCTACGAA		546
C S Q Y V K D I Y K Y L H I L E E Q Q P V R A N Y M Q G Y E		150
GTCAGTGAGCGCATGAGGGCACTCTCTGTTGACTGGCTTGTCCAAGTCCACTCCAGGTTCCAGCTGCTGCAGGAGACCCCTGTACCTCACT		636
V T E M R A L L V D W L V Q V H S R F Q L L Q E T L Y L T		180
GTGGCCATCTGGACCGCTTCTGCAAGTCCACCCGGTGGAGCGGTGGAAGCTGCAGCTCGTGGCGTCCAGCCATGCTGGTGGCGCTGC		726
V A I L D R F L Q V H P V S R R K L Q L V G V T A M L V A C		210
AAGTATGAGGAGATGTACGCTCCTGAAGTGGGGGACTTGGCTACATCACAGACAAGCCTTCAAAAATCTCAGATTGTGGAGATGGAG		816
K Y E E M Y A P E V G D F A Y I T D N A F T K S Q I V E M E		240
CAAGTATCTCAGGAGCCTCAGTTTCCAGCTGGGCGGCCCTCCCGTCCACTTCTCAGGCGAGCCACAAAGTTGCCGGCGTGAC		906
Q V I R S L S F Q L G R P L P L H <u>F L R R A T K</u> V A G C A		270
GTGGAGAAGCAGCTCTGGCAAAGTACCTGATGGAGCTGACCCCTCTGGACTACACATGTTGCAATTACCGGCCCTCTGAGGTTGTGCT		996
V E K H T L A K Y L M E L T L L D Y H M V H Y R P S E V A A		300
GCAGCGCTGTGTCTGCTCAGCTGCTGCTGATGGCTGCCATGCTGCTCACACAGCAGCAGTATTCACCTACGAAGAGCAGCATCTA		1086
A A L C L S Q L L L D G L P W S L T Q Q Q Y S T Y E E Q H L		330
AAACCCATCATGCAGCAGATGGCCAAAGACGTGGTGTGCTGCTGAACGAGGCGAGAACAAGTTCTTGGCGGTGAAGAAGTACTCCAGC		1176
K P I M Q H M A K N V V L V N E G R T K F L A V K K Y S S		360
AGCAAACTGATGAAGATCAGCCTCATTTCTCAGCTCAACTCTTCAACAGTCAAGACCATGGCGGATGCTCTGCACGATCACTGAACACA		1266
S K L M K I S L I P Q L N S S T V K T M A D A L H D H *		387
TTCTTAATGCTCTGGACTTTTAATTGCACTTTTAAATTTTAAAGACCTTAAAGGGAGGACTTGTCTGTCTCAACATGTACAAA		1356
TTGTATGGAGCATTTCTGTGGCGCAAGGAGCTACAGATGTTCAACTGTCTATTTTAAATAAATCTGAGTTTAAACAGTAAAAA		1446
AAAAA		

Fig. 1. Nucleotide and amino acid sequences of cDNAs encoding cyclins B1 and B2 isolated from medaka testis. The destruction box and the FLRRXSK motif are underlined and double-underlined, respectively. The sequence data appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB030069 (cyclin B1) and AB030070 (cyclin B2). The sequence data of cyclin B1 (AB030071) and cyclin B2 (AB030072) isolated from a cDNA library from medaka embryos (Iwamatsu stages 26–27, 1994; the library is a generous gift from Dr. Shigeki Yasumasu, Sophia University) are also available in the databases. They are probably splice variants different from those isolated from the testis.

After deparaffinization and rehydration, the slides were immersed in 10 mM citrate buffer (pH 6.0), heated in a microwave oven for 10 min, and allowed to cool for 15 min. The slides were washed in distilled water and TBS (20 mM Tris, 150 mM NaCl, pH 7.5) each 2 times for 5 min and treated with blocking solution (5% dry milk and 0.1% Triton X-100 in TBS) for 1 hr. After incubation with the affinity-purified anti-cyclin B1 or B2 polyclonal antibodies at 1:10 dilution, the slides were stained with a VECTASTAIN ABC-AP kit using Vector Red substrate (Vector Lab.).

RESULTS

Cloning and sequence analysis of medaka cyclins B1 and B2

The longest cDNA clone screened with the medaka cyclin B1 PCR product as a probe was 1662 bp and contained an open reading frame consisting of 404 amino acids (DDBJ/EMBL/GenBank Accession No. AB030069). The deduced amino acid sequence had the destruction box and the FLRRXSK motif (Fig. 1), and showed 69% homology to goldfish cyclin B1 (Hirai *et al.*, 1992) and 63% to *Xenopus* cyclin B1 (Minshull *et al.*, 1989), but lower homology (49%) was scored for *Xenopus* cyclin B2 (Minshull *et al.*, 1989).

The longest cDNA clone isolated with the medaka cyclin B2 PCR product was 1456 bp and encoded an open reading

frame of 387 amino acids (Accession No. AB030070). The deduced sequence had the destruction box, in which a leucine was substituted for an isoleucine, and the sequence FLRRATK similar to the FLRRXSK motif (Fig. 1). The amino acid sequence showed 56% homology to *Xenopus* cyclin B2 but lower homology (51%) to the cyclin B1 counterpart. Northern blotting showed that the lengths of cyclin B1 and B2 mRNAs expressed in medaka testis were 1.65 and 1.45 kb, respectively (data not shown), indicating that the clones isolated in this study are nearly the full-length of cDNAs encoding medaka testis cyclins B1 and B2.

In situ hybridization analysis of medaka testis

Expression of cyclins B1 and B2 at the mRNA level was investigated by using DIG-labeled antisense RNA probes (Figs. 2, 3). No signal was seen in the control sections hybridized with the sense probes (Fig. 2A, C). Cyclin B1 and B2 mRNAs were expressed in all stages of spermatogenic cells except for spermatozoa, although the expression levels varied according to the stages. The mRNA expression of both cyclins B1 and B2 was low in spermatogonia (Fig. 3B, C) and almost undetectable in spermatogonia at later anaphase and in primary spermatocytes at early prophase (data not shown, cf. Fig. 7).

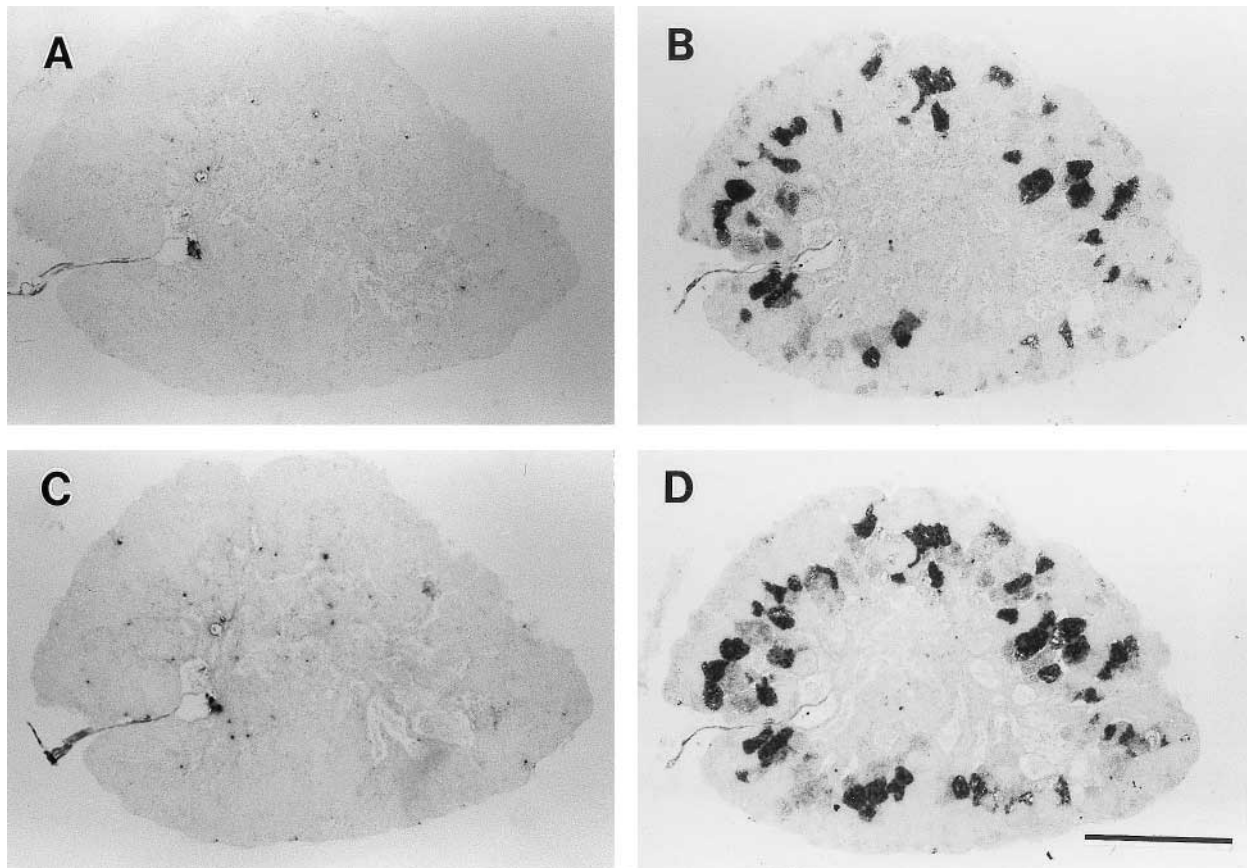


Fig. 2. *In situ* hybridization of medaka testis with DIG-labeled cyclin B1 (A, B) and cyclin B2 (C, D) RNA probes. No signal was detected with the sense probes of cyclins B1 (A) and B2 (C). With the antisense probes, however, different mRNA expression patterns were seen between cyclins B1 (B) and B2 (D). Scale, 300 μ m.

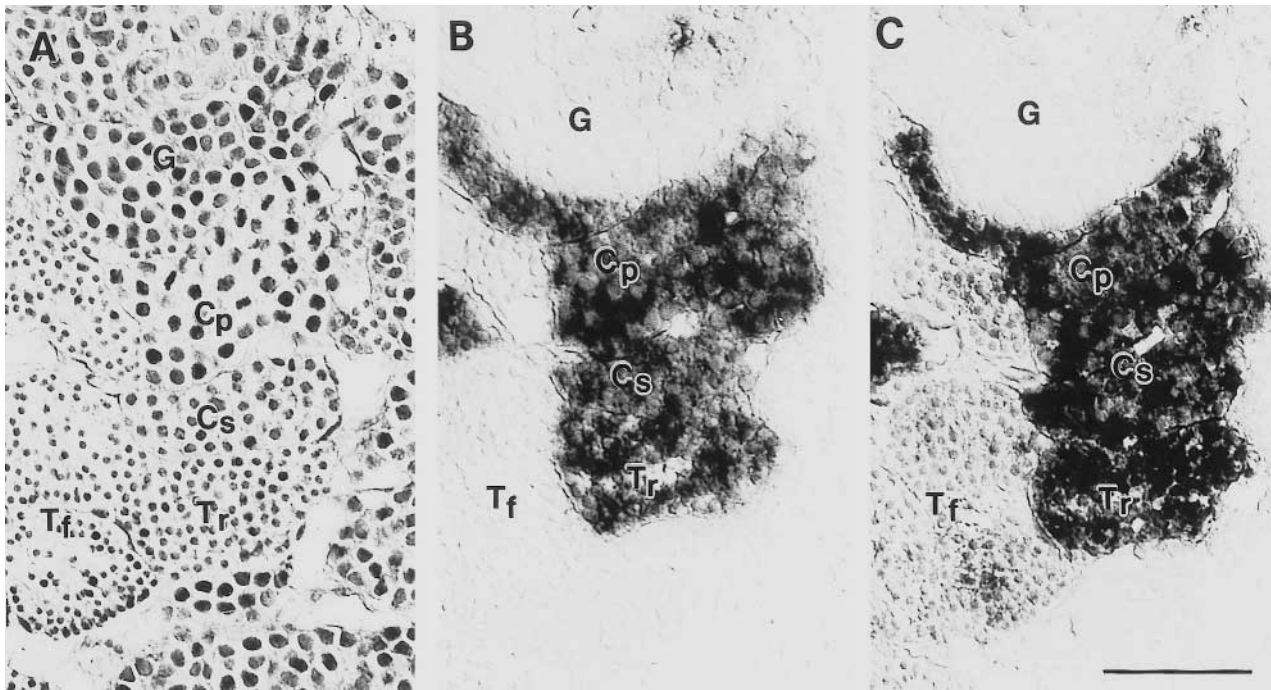


Fig. 3. Cyclin B1 and B2 mRNA expression during medaka spermatogenesis. Sections were hybridized with the antisense cyclin B1 (**B**) and B2 (**C**) RNA probes. Spermatogenic stages were assessed by the adjacent section stained with hematoxylin and eosin (**A**) according to Shimizu *et al.* (1997). Cyclin B1 mRNA is expressed weakly in spermatogonia (G) but strongly in primary (Cp) and secondary (Cs) spermatocytes at and after the pachytene stage and in round spermatids (Tr). The expression patterns of cyclin B2 mRNA are similar to those of cyclin B1 mRNA, except that cyclin B2, but not B1, is expressed in flagellum-elongating spermatids (Tf). Scale, 30 μ m.

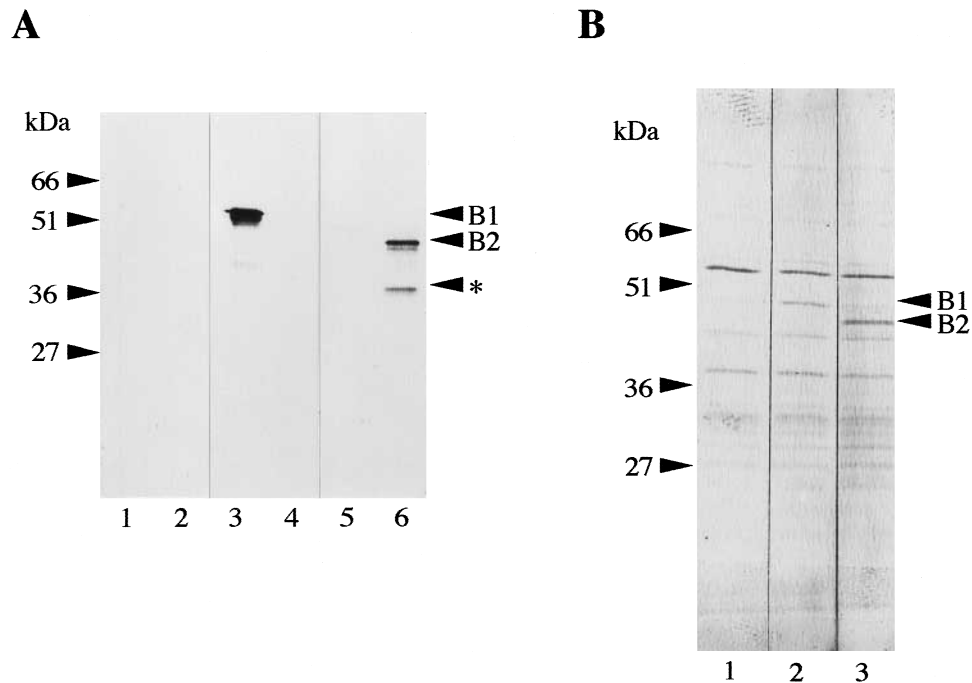


Fig. 4. Characterization of the antibodies raised against the recombinant medaka cyclin B1 and B2 proteins. (**A**) Immunoblotting of the crude lysates from *E. coli* that expresses medaka cyclin B1 (T7- Δ 0-cyclin B1, lanes 1, 3 and 5) or cyclin B2 (T7- Δ 25-cyclin B2, lanes 2, 4 and 6), using anti-cyclin B1 antibody (lanes 3, 4) and anti-cyclin B2 antibody (lanes 5, 6). No signal was found in the control without the first antibody (lanes 1, 2). The anti-B1 and -B2 antibodies recognize the recombinant cyclin B1 and B2 proteins, respectively. The band recognized by the anti-B2 antibody (indicated by an asterisk) is probably a degradation product from the intact cyclin B2 molecule. (**B**) Immunoblotting of the extracts obtained from medaka testis with anti-cyclin B1 antibody (lane 2) and anti-cyclin B2 antibody (lane 3), showing the anti-cyclin B1-reactive 49.5 kDa protein and the anti-cyclin B2-reactive 48 kDa protein, respectively. These proteins were not detected in the control without the first antibody (lane 1).

mRNA expression levels of cyclins B1 and B2 abruptly increased in primary spermatocytes at the pachytene stage (characterized by the presence of thick thread-like chromosomes in the nucleus, Fig. 3B, C). Some cysts containing the pachytene spermatocytes expressed a high level of mRNA, while others containing similar cells did not express mRNA at

all, indicating that abrupt expression occurs during the pachytene stage. The mRNA expression levels of both cyclins B1 and B2 remained high until the formation of round spermatids via secondary spermatocytes, and they decreased thereafter (Fig. 3B, C). In flagellum-elongating spermatids, cyclin B2 mRNA was still expressed at low levels, while cyclin

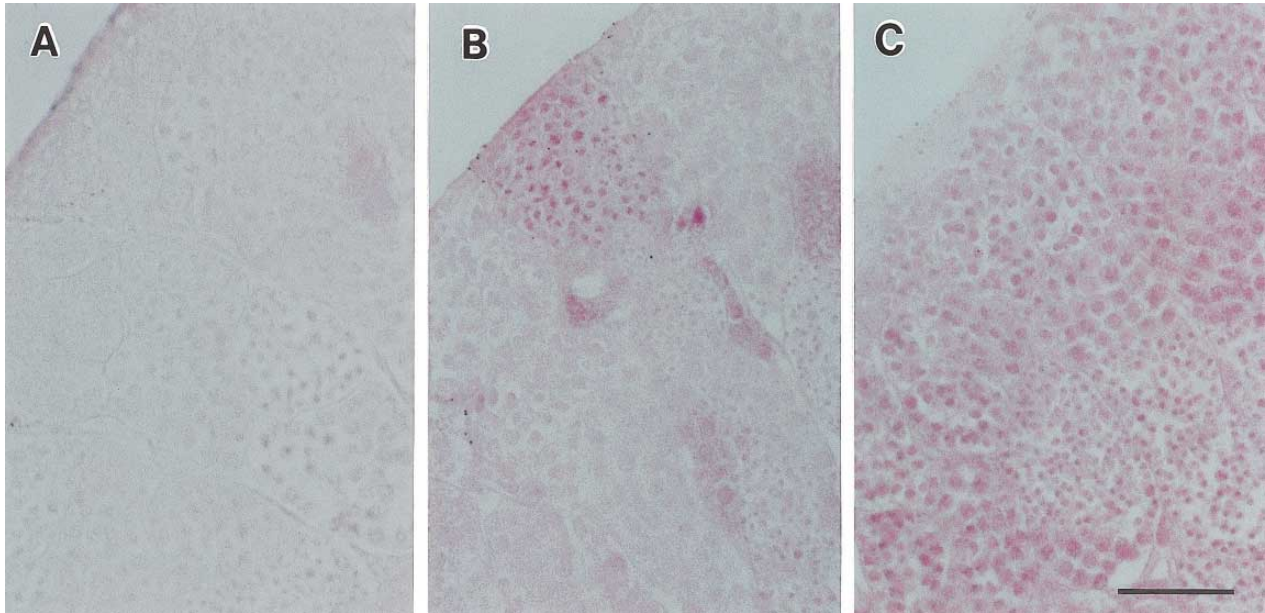


Fig. 5. Immunocytological analysis of medaka testis with anti-cyclin B1 antibody (**B**) and anti-cyclin B2 (**C**) antibody. Positive signals appear in red. No signal was observed in the control section without the first antibody (**A**). Note the difference in the expression patterns between cyclin B1 and B2 proteins. scale, 30 μ m.

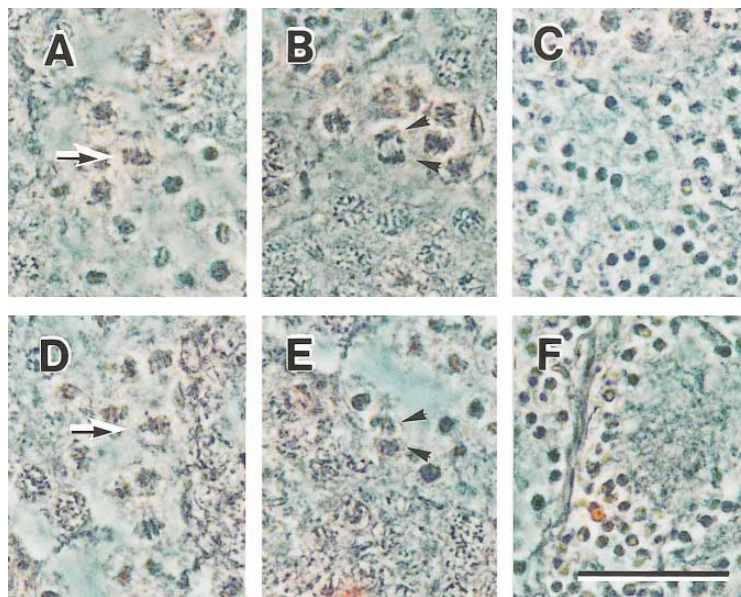


Fig. 6. Protein expression of cyclin B1 (**A–C**) and cyclin B2 (**D–F**) in medaka spermatogenic cells. Observations were carried out with a phase-contrast microscope to visualize the nuclear morphology. Scale, 20 μ m. **A, D:** Primary spermatocytes at metaphase are indicated by arrows. **B, E:** Primary spermatocytes at anaphase are indicated by arrowheads. **C, F:** Spermatids. Cyclin B1 protein is expressed in primary and secondary spermatocytes at prophase (at and after the pachytene stage) and metaphase, then disappears at anaphase (**B**). No expression was seen in spermatids (**C**). On the other hand, cyclin B2 is expressed throughout spermatogenesis, even in spermatocytes at anaphase (**E**) and spermatids (**F**).

B1 was not (Fig. 3C). Thus, the mRNA expression of cyclin B2 lasted longer than that of cyclin B1.

Immunocytological observation of cyclins B1 and B2 in medaka testis

The specificity of newly produced antibodies against the recombinant medaka cyclin B1 (T7- Δ 0-cyclin B1) and cyclin B2 (GST- Δ 25-cyclin B2) was confirmed by immunoblotting (Fig. 4). The anti-cyclin B1 antibody recognized the antigenic cyclin B1 protein but not cyclin B2. Conversely, the anti-cyclin B2 antibody recognized the recombinant cyclin B2 protein without GST (T7- Δ 25-cyclin B2) but not cyclin B1 (Fig. 4A). When medaka testis extracts were immunoblotted with the anti-cyclin B1 and B2 antibodies, 49.5 and 48 kDa proteins were specifically detected, respectively (Fig. 4B). Although the values of the apparent molecular mass of the immunoreactive proteins (native cyclins B1 and B2) are not perfectly consistent with those deduced from the cDNA sequences (45 kDa for cyclin B1 and 43 kDa for cyclin B2), such a disagreement is a general characteristic of cyclins (Gautier *et al.*, 1990).

The expression patterns of cyclin B1 and B2 proteins were investigated immunocytologically (Figs. 5, 6). Cyclin B1 protein was expressed in spermatogonia at prophase and metaphase and disappeared at anaphase. In primary spermatocytes, the protein was absent at early prophase but was strongly expressed thereafter (at and after the pachytene stage to metaphase) and disappeared at anaphase (Fig. 6 A, B). Cyclin B1 protein appeared again in secondary spermatocytes with similar changes according to the cell cycle. Cyclin B1 protein was not detected in spermatids or spermatozoa (Fig. 6C).

In striking contrast to the expression patterns of cyclin B1 protein, cyclin B2 protein was continuously expressed throughout spermatogenesis (Figs. 5C, 6D-F) without a transient disappearance at each anaphase (Fig. 6E). Cyclin B2 protein existed even in spermatids and spermatozoa (Fig. 6F), which had undergone meiosis and terminally differentiated.

DISCUSSION

In this report, we have described the molecular cloning of cDNAs encoding medaka cyclins B1 and B2 and their expression patterns at mRNA and protein levels. Fig. 7 summarizes the results obtained in this study. Both cyclin B1 and B2 mRNAs were expressed from spermatogonia (although low in these cells) to round spermatids through spermatocytes, except for spermatogonia at later anaphase and primary spermatocytes at early prophase (until the pachytene stage), during which mRNA expression was not observed. Cyclin B2 mRNA expression continued in flagellum-elongating spermatids. Cyclin B1 protein was expressed only in spermatogonia and spermatocytes at prophase and metaphase, with a transient disappearance at each anaphase, whereas cyclin B2 protein was continuously expressed throughout spermatogenesis including spermatozoa.

Differences between cyclin B expressions at mRNA and protein levels

The expression patterns of cyclin B proteins did not agree with those of mRNAs. Medaka spermatogenic cells strongly expressed cyclin B1 and B2 mRNAs at the spermatocyte stage, whereas their protein expression did not apparently

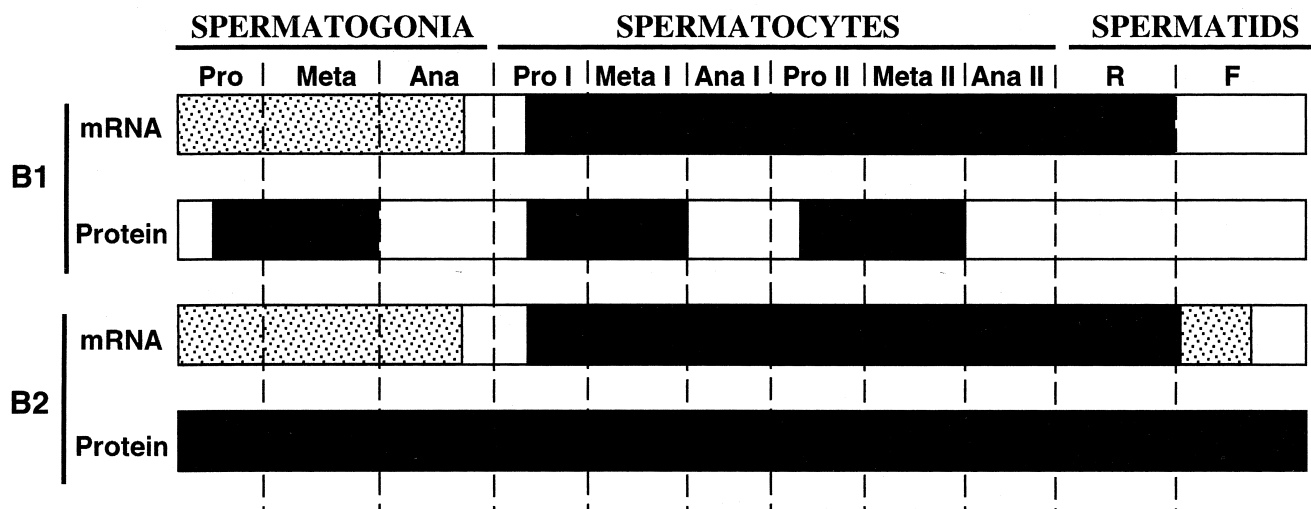


Fig. 7. Summary of the expression patterns of medaka cyclins B1 and B2 at mRNA and protein levels. The open and the solid bars show the absence and the presence of the products, respectively. The shaded bars indicate mRNA expression that is less than that indicated by the solid bars. The abrupt mRNA expression of both cyclins B1 and B2 occurs in the pachytene spermatocytes in the first meiotic prophase (Pro I) and lasts until the round spermatids for cyclin B1 and the flagellum-elongating spermatid for cyclin B2. Cyclin B1 protein exists only in spermatogonia and spermatocytes around metaphase, while cyclin B2 protein is present throughout the spermatogenic stages, even in spermatozoa (although not shown in this figure). Pro, prophase; Meta, metaphase; Ana, anaphase; R, round spermatids; F, flagellum-elongating spermatids. The numbers following the abbreviations (I and II) indicate the first and the second meiotic divisions, respectively.

increase in this stage. Cyclin B1 mRNA was expressed in round spermatids, whereas its protein was absent in these cells (Fig. 7). Similar discrepancy between mRNA and protein expressions of cyclin B has been reported in mouse spermatogenesis (Brandeis *et al.*, 1998; Chapman and Wolgemuth, 1994). It is clear at least in medaka that the protein expression during the later phases of spermatogenesis (at and after the spermatocyte stage) is mainly controlled at translational levels, since primary spermatocytes isolated from medaka testis are able to transform into spermatozoa under inhibited transcription (Shimizu *et al.*, 2000).

During the early phase of spermatogenesis, there must be critical points for cell differentiation, including the acquisition of competence to enter meiosis as suggested in eel spermatogonia (Miura *et al.*, 1997). In this context, it is of interest that cyclin B mRNA expression is not continuous during spermatogenesis but stops at the later stage of spermatogonia and restarts from primary spermatocytes at the pachytene stage (Fig. 7). The transient absence of cyclin B transcripts during later spermatogonia and the early primary spermatocytes may imply the reorganization of the control systems of protein expression from those suitable for mitotically dividing cells to those suitable for meiotically dividing cells, and after the onset of new control systems that depend mainly on the translational control, medaka spermatogenic cells (primary spermatocytes) seem to automatically continue spermatogenesis. Molecular bases of this restriction point remain to be elucidated.

The presence of mRNA without its protein product is commonly observed in animal eggs, which undergo meiosis like spermatogenic cells (Yamashita *et al.*, 2000). Namely, mRNAs are transcribed and stored in the oocytes as maternal mRNAs, but not translated immediately, during vitellogenesis. Gametogenic cells tend to employ translational control, instead of transcriptional control, for regulating protein expressions. This characteristic is thought to be related to the destiny of gametogenic cells: the oocyte must store maternal mRNAs (as well as proteins), and the spermatozoon must have a condensed nucleus in which transcription is inactive.

Distinct functions of cyclins B1 and B2

In many species, at least two different types of cyclin B are present, but their functional differences remain a mystery (Yoshitome *et al.*, 1998). We have shown here that cyclin B1 protein is expressed in spermatogonia and spermatocytes with a transient disappearance at anaphase, whereas cyclin B2 protein is expressed in these cells even at anaphase (Fig. 7). The different expression patterns between cyclins B1 and B2 suggest that they have distinct functions during medaka spermatogenesis. The transition from metaphase to anaphase is induced by the ubiquitin-mediated degradation of cyclin B proteins, which in turn leads to the inactivation of MPF (Peters, 1998; Tokumoto, 1999). Accordingly, the continuous presence of cyclin B2 at the metaphase-anaphase transition suggests that this protein does not function at least in this process of meiotic cell cycle in medaka spermatogenesis, in striking con-

trast to cyclin B1, which is thought to be involved in cell cycle regulation. However, we must await biochemical studies, including studies on changes in the activities of cyclin B1/Cdc2 MPF and cyclin B2/Cdc2 MPF during spermatogenesis, to verify this hypothesis, although such studies are difficult because of the asynchronous spermatogenesis in medaka testis as a whole and because of the limited number of medaka spermatogenic cells available for biochemical analyses.

In this study, we have demonstrated the existence of cyclin B2 protein in medaka spermatids that underwent meiosis. It has also been reported that cyclin B2 protein is present in differentiated chicken lens fiber cells (Gao *et al.*, 1995) and that cyclin B1 protein is expressed in rat spermatids with peak expression at the formation of chromosomal vesicles (Gromoll *et al.*, 1997). Furthermore, the histone H1 kinase activity, an indicator of MPF activity, is detectable in terminally differentiated non-dividing cells (Gao and Zelenka, 1997), including spermatids of the rat (Gromoll *et al.*, 1997) and newt (M. Ito, M. Yamashita, Y. Matsuda, T. Yamamoto and S.-I. Abe, unpublished). These findings suggest that both cyclins B1 and B2 have functions other than cell cycle regulation. This notion is consistent with the behavior of medaka testis cyclin B2 protein irrespective of the cell cycle but inconsistent with the behavior of cyclin B1 protein in accordance with the cell cycle. In addition, neither cyclin B1 nor B2 is expressed in mouse spermatids despite their expression in spermatocytes at high levels, suggesting that their functions are restricted to the meiotic phases before spermiogenesis (Brandeis *et al.*, 1998). The above-mentioned discrepancy might be due to a species-difference in the functions of cyclin B subtypes. Another possible explanation is that the nomenclature based solely on the sequence similarity does not actually reflect the functional homologue. For example, rat testis cyclin B1, the expression of which peaks at post meiotic spermatids (Gromoll *et al.*, 1997) may not be a real functional homologue of medaka testis cyclin B1, which exhibits cell cycle-dependent expression, or rather, rat cyclin B1 seems to resemble medaka cyclin B2. Similarly, mouse cyclin B2 may not correspond to medaka cyclin B2. To verify this hypothesis, we must accumulate detailed biochemical data on each cyclin B molecule, such as data on the differences in kinase activity and substrate specificity of Cdc2 coupled with different subtypes of cyclin B. Further investigations in which the function of each cyclin molecule is manipulated by introducing mRNA or antisense RNA into spermatogenic cells cultured *in vitro* (Shimizu *et al.*, 1997) will also greatly contribute to clarification of the functions of cyclin B subtypes in spermatogenesis.

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