Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 323 (2004) 288–292
BBRC
www.elsevier.com/locate/ybbrc

The cloning of cyclin B3 and its gene expression during hormonally induced spermatogenesis in the teleost, *Anguilla japonica*

Hiroko Kajiura-Kobayashi[a,b], Tohru Kobayashi[a,b,c,*], Yoshitaka Nagahama[a,b]

[a] *Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan*
[b] *CREST, Kawaguchi, Saitama 332-0012, Japan*
[c] *National Research Institute of Aquaculture, Tamaki, Mie 516-0423, Japan*

Received 9 August 2004

Abstract

We cloned cyclin B1, B2, and B3 cDNAs from the eel testis. Northern blot analysis indicated that these cyclin B mRNAs were expressed and increased from day 3 onward after the hormonal induction of spermatogenesis, and that cyclin B3 was most dominantly expressed during spermatogenesis. In situ hybridization showed that cyclin B1 and B2 were present from the spermatogonium stage to the spermatocyte stage. On the other hand, cyclin B3 mRNA was present only in spermatogonia. Although mouse cyclin B3 is expressed specifically in the early meiotic prophase, these results indicate that eel cyclin B3 expression is limited during spermatogenesis to spermatogonia, but is not present in spermatocytes. These facts together suggest that eel cyclin B3 is specifically involved in spermatogonial proliferation (mitosis), but not in meiosis.

Keywords: Cyclin B1; Cyclin B2; Cyclin B3; Spermatogenesis; Spermatogonia; Mitosis; Gene expression; Teleost; Eel

The identification of many gene products involved in controlling the mitotic and meiotic cell cycles of several model organisms has greatly facilitated our understanding of cell cycle regulation. The B-type cyclins associate with the 34 kDa Cdc2 serine/threonine protein kinase to form a complex known as MPF, maturation-, or M-phase promoting factor [1–3]. Although MPF activity during oogenesis has been well studied, few studies have been done on MPF activity during the mitotic and meiotic cell cycles in the process of spermatogenesis. At present, the expression by in situ hybridization of two types of cyclin B, cyclin B1 and B2, and MPF activity have been reported in the mammalian testis [4].

Originally cloned from a chicken, cyclin B3 is expressed at very low levels in virally transformed hematopoietic cell lines and the early embryonic stages of the chicken. When chicken cyclin B3 is introduced into HeLa cells, it localizes in the nucleus and forms complexes with cdc2 and cdk2 [5]. In *Drosophila*, cyclin B3 is expressed in both mitotic and meiotic cells. Cyclin B3 is essential for fertility, but, unlike cyclin B1 deficiency, infertility in cyclin B3-deficient flies is restricted to the female germ line and does not appear to involve defects in the ovary structure or in meiosis I entry [6,7]. Mammalian cyclin B3 displayed nuclear localization and degraded upon anaphase entry following cyclin B1 degradation, when cyclin B3 was introduced into mitotic cells. However, endogenous cyclin B3 could not be detected in cell lines from a wide variety of cell types. Recently, it was reported that mammalian cyclin B3 mRNA and protein were expressed in prepachytene spermatocytes, specifically during the leptotene and zygotene stages, and in nests of female oocytes during embryonic development [8].

In lower vertebrates, the induction and completion of spermatogenesis in vivo and in vitro have been achieved [9,10]. In particular, the Japanese eel testis provides an

* Corresponding author. Fax: +81 596 58 6413.
E-mail address: tohrukob@affrc.go.jp (T. Kobayashi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.08.097

excellent model for the clarification of the molecular mechanisms involved in the initiation of spermatogenesis, because only stem cell-type spermatogonia (type A spermatogonia) are present in the testes of the cultivated eel, and spermatogenesis can be induced by hormones in vivo and in vitro [11–13]. Although many studies dealing with B-type cyclins were done during oocyte maturation in non-mammalian vertebrates [14], to date no expression profiles of B-type cyclins, especially cyclin B3, after the initiation of spermatogenesis have been reported in non-mammalian vertebrates. Here, we report on the isolation of B-type cyclin cDNA (cyclin B1, B2, and B3) and the gene expression of each B-type cyclin during hormonally induced spermatogenesis in a teleost fish, the Japanese eel.

Materials and methods

Animals. Japanese eels, *Anguilla japonica*, were purchased from a commercial eel supplier (Hekinan City, Aichi Prefecture, Japan) and were kept in recirculating freshwater tanks with a capacity of 500 L at 22 ± 1 °C until use.

Isolation of cyclin B1, B2, and B3 cDNAs. In order to isolate cyclin B homologs, degenerate oligonucleotide primers used in PCR experiments were designed. The two primers corresponded to the conserved amino acid sequences of cyclin B, MR(A/D/L)(I/L)L(I/V)(N/P)W, and FLRRASK, respectively. RT-PCR has been done according to a previous report [15]. After subcloning, the plasmid DNA showing similarity to the conserved domain of B-type cyclins was used to isolate full-length cDNA clones from the cDNA library as a probe. After screening, the resulting phagemids containing the cDNA of interest were then inserted at the *Eco*RI sites within the pBluescript SK(−) plasmid. Isolated phagemids were sequenced using the double-stranded sequencing procedure for the AmpliTaq Sequencing Kit. DNA sequence analysis was also performed on an Applied Biosystems Model 377A DNA sequencer.

Phylogenetic analysis. A phylogenetic tree of amino acid sequences was constructed by the neighbor-joining method [16] using PHYLIP 3.573c. The evolutionary distance between two aligned amino acid sequences was measured by use of the Dayhoff PAM matrix. The robustness of the phylogenetic hypothesis was tested by bootstrapping [17]. In the present study, all bootstrap analyses of amino acids involved 1000 replications of the data.

Northern blot analysis. Poly(A)$^+$ RNA was prepared from the testes of untreated eels (0 day) and testes of hCG treated eels (1, 3, 6, 9, 12, 15, and 18 days after injection). Poly(A)$^+$ RNA (2 μg) was separated by electrophoresis on a denaturing 1.1% agarose gel containing 6% formaldehyde. After electrophoresis, RNA was transferred onto Hybond-N$^+$, and hybridized at 65 °C overnight with an [α-^{32}P]dCTP-labeled PCR probe (10,000,000 cpm) prepared using cyclin B1, cyclin B2, or cyclin B3 cDNAs as templates. These PCR probes were amplified by specific primer sets for cyclin B1, B2, and B3, as described above. The membranes were washed twice at 65 °C for 30 min with 1× SSC containing 0.1% SDS, and analyzed with a BAS2000 Bio-Image Analyzer (Fuji, Japan).

In situ hybridization. In order to prepare templates for the in situ hybridization probes, the 5′ regions of cyclin B1, B2, and B3 cDNAs were amplified using specific primer sets. These 5′ regions of cyclin B1, B2, and B3 cDNAs were hybridized specifically with cyclin B1, B2, and B3 mRNA, respectively. Finally, using linearized cyclin B1, B2, and B3 cDNAs as templates, RNA probes were synthesized using a DIG RNA Labeling Kit (Roche, USA).

The testes were removed from eels at 6 and 18 days after a single injection of hCG, and were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4 °C overnight. The fixed tissues were immersed in 0.1 M PB for 30 min twice, dehydrated, and then embedded in paraffin wax. Sections were cut at a thickness of 5 μm. In situ hybridization was performed according to the protocol given in a previous report [18].

Transfection of B-type cyclins into HeLa cells. In order to characterize the B-type cyclins, pEGFP-cyclin B1, B2, or B3 was transfected into HeLa cells using FuGENE6 transfection reagent (Roche, USA). As a control, pEGFP without any B-type cyclin was transfected into HeLa cells. At 24–48 h after transfection, the transfected HeLa cells were observed under fluorescent microscopy.

Results

Eel cyclin B1, B2, and B3 sequences

The isolated cyclin B1 cDNA is 1396 bp long, containing a poly(A)$^+$ tail, with an open reading frame (ORF) encoding 403 amino acids (a.a.). The isolated cyclin B2 and B3 cDNAs were 1611 and 1843 bp long, respectively, each containing a poly(A)$^+$ tail, with an ORF encoding 394 and 404 a.a., respectively. Eel cyclin B1 displayed a higher similarity to B1 cyclins than B2 cyclins (*Xenopus* B1, 66.3%; mouse B1, 63.3%; goldfish B1, 76.0%; *Xenopus* B2, 52.6%; and mouse B2, 52.0%). Similarly, eel cyclin B2 displayed a higher similarity to B2 cyclins than B1 cyclins (*Xenopus* B2, 63.4%; mouse B2, 58.2%; *Xenopus* B1, 56.7%; and mouse B1, 58.2%). A region known as the ''destruction box,'' which is believed to be involved in ubiquitin-mediated proteolysis pathways, is found in the amino terminal end of both predicted proteins. This conserved region consists of nine amino acids, of which the consensus for the general structure of B-type cyclins is RxALGxIxN (RxxLxxxxN), followed by a region rich in lysine residues. There were two highly conserved regions among the cyclin B3 proteins from different organisms. The similarities were restricted to the termini and included a putative destruction box motif of the RxxFxxxxN type at the N terminus and a cyclin box motif at the C terminus. As indicated in a previous report [5], cyclin B3 was most similar to A- and B-type cyclins (Fig. 1). Although cyclin B3 also had nuclear localization signals (NLS: eel B3, 33–46; KRSPSSPQGAPKKR) in a similar fashion to cyclin B1 and B2, cytoplasmic retention signals (CRS) were not observed [19–21].

Localization of cyclin B3 transfected into HeLa cells

When pEGFP was transfected into HeLa cells, EGFP was observed in both nucleus and cytoplasm (Fig. 2A). Similarly, pEGFP-cyclin B1 and B2 were observed in the cytoplasm, although pEGFP-cyclin B1 had also localized in the nucleus (Figs. 2B and C). In contrast to the localization of cyclin B1 and B2, transfected

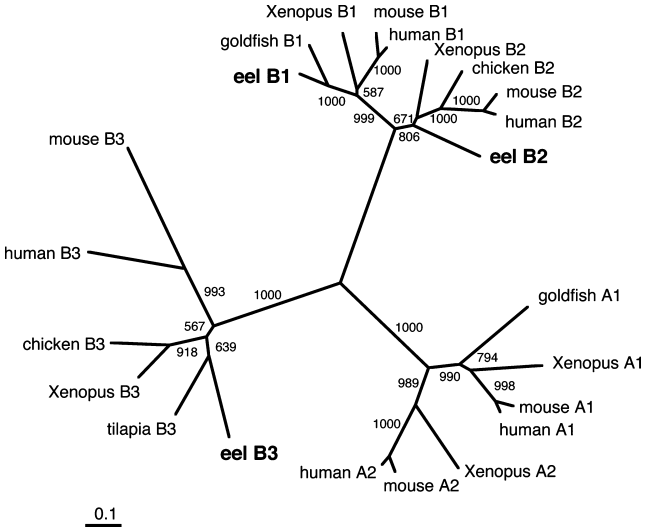

Fig. 1. Phylogenetic tree of the amino acid sequences of the cyclin B family. The tree was constructed by the neighbor-joining method. The numbers indicate bootstrap values from 1000 replicates. The horizontal lines indicate genetic distances. The GenBank accession numbers are: eel cyclin B1, AB183431; eel cyclin B2, AB183432; eel cyclin B3, AB183429; goldfish cyclin B1, S48758; human cyclin B1, BC006510; mouse cyclin B1, X64713; *Xenopus* cyclin B1, J03166; human cyclin B2, CR53327; mouse cyclin B2, X66032; chicken cyclin B2, X62531; *Xenopus* cyclin B2, J03167; human cyclin B3, AJ416459; mouse cyclin B3, AJ416459; chicken cyclin B3, X75757; *Xenopus* cyclin B3, AJ304990; tilapia cyclin B3, AB183430; goldfish cyclin A1, S79215; *Xenopus* cyclin A1, X53745; mouse cyclin A1, X84311; human A1, BC036346; *Xenopus* cyclin A2, X85746; mouse cyclin A2, Z26580; and human A2, CR407692.

pEGFP-cyclin B3 had localized only in the nucleus (Figs. 2D and E).

Northern blot analysis

The patterns of cyclin B1, B2, and B3 expression during spermatogenesis in the eel testis were examined by Northern blot hybridization. Poly(A)$^+$ RNA was prepared from intact eels and eels 1, 3, 6, 9, 12, 15, and 18 days after hormonal induction of spermatogenesis (Fig. 3). A single 1.7-kb band, corresponding to cyclin B1, cyclin B2 transcripts 2.0 kb long, and cyclin B3 transcripts 1.8 kb long, was detected in the eel testis 1 day after hCG treatment. These B-type cyclin transcripts increased with the progression of spermatogenesis after injection and reached peak levels in the eel testis 6 days after injection. These results suggest that all B-type cyclins examined are expressed from the spermatogonium stage.

In situ hybridization

The distribution of B-type cyclin mRNA in testes during spermatogenesis was examined in testis at 6 and 18 days after the hormonal initiation by in situ hybridization. Cyclin B1 and B2 mRNAs were present

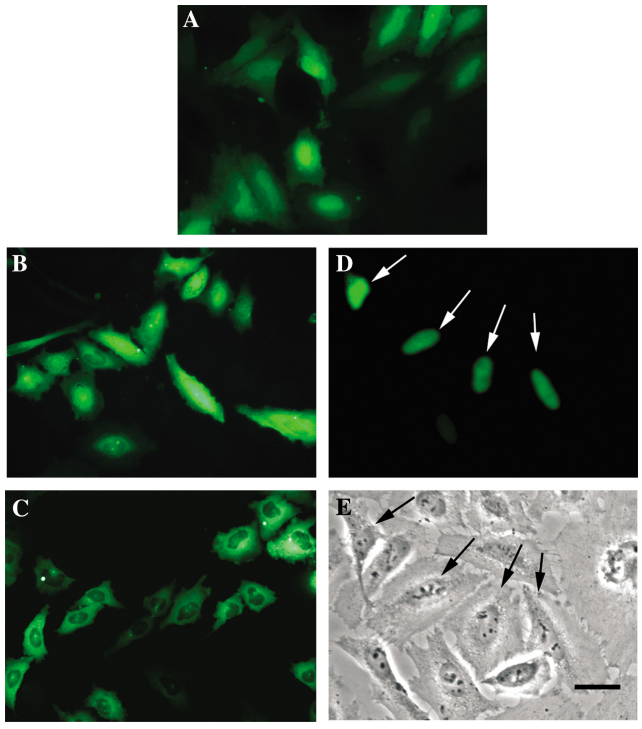

Fig. 2. Eel cyclin B3 accumulates in the nucleus of mitotic cells. HeLa cells were transfected with pEGFP vectors expressing N-terminally tagged cyclin B1, B2, or B3 as described under Materials and methods. This is a representative example of more than 50 cells examined for each construct. (A) EGFP, (B) EGFP-cyclin B1, (C) EGFP-cyclin B2, (D) EGFP-cyclin B3, and (E) light field of section C. Arrows indicate cells expressing EGFP-cyclin B3. Scale bar: 20 μm.

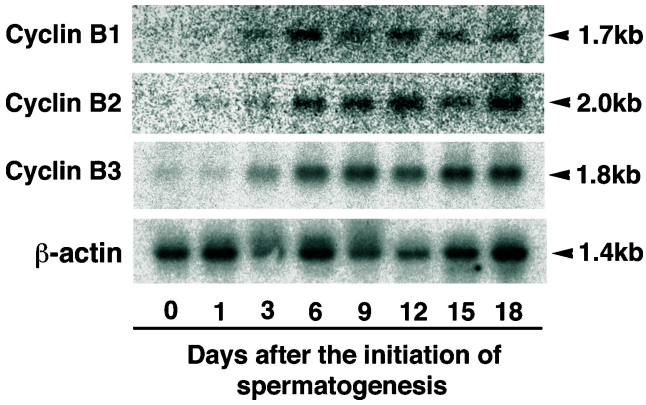

Fig. 3. Expression of B-type cyclin mRNA during hormonally induced spermatogenesis. Poly(A)$^+$ RNA was extracted from the testes sampled on day 0 (intact eels) and 1, 3, 6, 9, 12, 15, and 18 after the hormonal induction of spermatogenesis, and 2 μg of each sample was subjected to electrophoresis.

from the spermatogonium stage to the spermatocyte and testicular somatic cell stage, including sertoli cells (Figs. 4A, B, D, and E). Similarly, cyclin B3 mRNA was present in spermatogonia. However, the signals for cyclin B3 mRNA were not detected in the spermatocyte and spermatid stage (Figs. 4C and F). These results

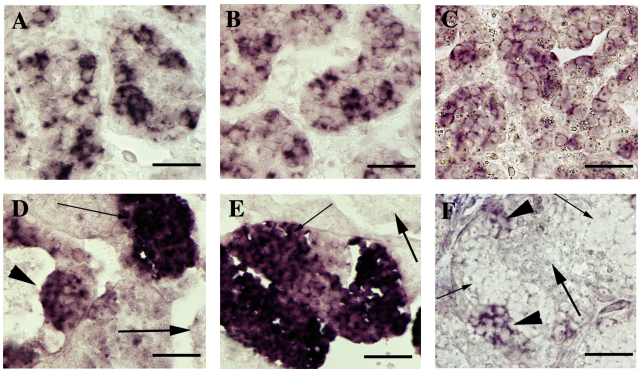

Fig. 4. In situ hybridization of B-type cyclins in the testis before and after the induction of spermatogenesis. (A,D) In situ hybridization of cyclin B1. (B,E) In situ hybridization of cyclin B2. (C,F) In situ hybridization of cyclin B3. (A–C) Six days after the hormonal induction of spermatogenesis. Most progressing spermatogenic cells are type-B spermatogonia [11,13]. Signals for cyclin B1, B2, and B3 mRNAs had localized in the spermatogonia. (D–F) Eighteen days after the hormonal induction of spermatogenesis. All stages of spermatogenesis are seen [11,13]. Signals for cyclin B1 and B2 were seen from the spermatogonium stage to the spermatocyte stage. In contrast, signals for cyclin B3 were seen in spermatogonia specifically. Arrowheads, type-B spermatogonia; small arrows, spermatocytes; large arrows, spermatids. Scale bar: 30 μm.

suggest that cyclin B3 mRNA expression is limited to the spermatogonium stage but not present in the early meiotic prophase, such as in mice [8].

Discussion

The present study indicates that the conservation of cyclin B3 sequences has been demonstrated. A comparison of cyclin B3 with other vertebrate cyclins shows that it is slightly more closely related to B- than A-type cyclins (Fig. 1). Regarding cellular localization, eel cyclin B1 and B2 displayed cytoplasmic localization, in agreement with previous reports [22–24]. Furthermore, eel cyclin B3 proteins localized in the nucleus in mitotic cell line cells, in concordance with previous reports [5,8]. These results suggest the conservation of the structure and nature of cyclin B3 in vertebrates.

Although the details of endogenous cyclin B3 localization were unclear, a recent study on mice demonstrates that cyclin B3 is expressed during a short period of meiosis, beginning at the onset of the first meiotic prophase, and ending at the pachytene stage. To date, however, its function during the meiotic prophase has remained obscure [8]. In non-mammalian vertebrates, no expression profiles for cyclin B3 have been examined in spermatogenic cells, although cloning of cyclin B3 has been done for several non-mammalian vertebrates. This study reports the expression profiles for cyclin B3 in non-mammalian spermatogenic cells for the first time. Our present study has revealed that eel cyclin B3 displays gonad-specific expression in a manner similar to mouse cyclin. However, eel cyclin B3 expression was limited to the spermatogonium stage of spermatogenesis. The main difference in the characteristics of mouse cyclin B3 and eel cyclin B3 consisted in their expression sites in the testis. Recently, we also cloned a cyclin B3 homolog from a teleost fish, *Nile tilapia* (see Fig. 1), and its expression profile indicated that Tilapia cyclin B3 was expressed in spermatogonia, similar to eel cyclin B3 (data not shown). These facts may imply that teleost cyclin B3 is specifically expressed in spermatogonia but not in spermatocytes, in contrast to mammalian cyclin B3. It is interesting whether the role of cyclin B3 is conserved in mammals and fish.

Nguyen et al. [8] show that enforced expression of cyclin B3 affects the anaphase exit and that cyclin B3 could act with cdk2, with undetectable histone H1 kinase activity, suggesting the possibility of cdk-independent mechanisms or mechanisms in which the cyclin interferes with the regulated proteolysis of another component. Based on these results, they arrived at the hypothesis that cyclin B3 might play a role in the formation of the synaptonemal complex, and that cyclin B3 expression might prevent precocious pachytene entry. In this study, unfortunately, we did not examine the characteristics of cyclin B3 at the protein level, although the expression profiles of cyclin B3 in fish are unique as compared with those of mammalian cyclin B3. Further studies will be necessary in order to clarify the biochemical characteristics of cyclin B3 in spermatogenic cells in non-mammalian vertebrates, including teleosts.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture, Japan, CREST, JSTs and Bio Design Program from the Ministry of Agriculture, Forestry and Fisheries, Japan.

References

[1] W.G. Dunphy, L. Brizuela, D. Beach, J. Newport, The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis, Cell 54 (1988) 423–431.
[2] J. Gautier, C. Norbury, M. Lohka, P. Nurse, J. Maller, Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene cdc2+, Cell 54 (1988) 433–439.
[3] J.C. Labbe, A. Picard, G. Peaucellier, J.C. Cavadore, P. Nurse, M. Doree, Purification of MPF from starfish: identification as the H1 histone kinase p34cdc2 and a possible mechanism for its periodic activation, Cell 57 (1988) 253–263.
[4] D.J. Wolgemuth, Insights into regulation of the mammalian cell cycle from studies on spermatogenesis using genetic approaches in animal models, Cytogenet. Genome Res. 103 (2003) 256–266.

[5] P. Gallant, E.A. Nigg, Identification of a novel vertebrate cyclin: cyclin B3 shares properties with both A- and B-type cyclins, EMBO J. 13 (1994) 595–605.
[6] H.W. Jacobs, J.A. Knoblich, C.F. Lehner, *Drosophila* cyclin B3 is required for female fertility and is dispensable for mitosis like cyclin B, Genes Dev. 12 (1998) 3741–3751.
[7] D.H. Parry, P.H. O'Farrell, The schedule of destruction of three mitotic cyclins can dictate the timing of events during exit from mitosis, Curr. Biol. 11 (2001) 671–683.
[8] T.B. Nguyen, K. Manova, P. Capodieci, C. Lindon, S. Bottega, X.-Y. Wang, J. Refik-Rogers, J. Pines, D.J. Wolgemuth, A. Koff, Characterization and expression of mammalian cyclin B3, a prepachytene meiotic cyclin, J. Biol. Chem. 277 (2002) 41960–41969.
[9] S. Abe, Differentiation of spermatogenic cells from vertebrates in vitro, Int. Rev. Cytol. 109 (1987) 159–209.
[10] Y. Nagahama, T. Miura, T. Kobayashi, The onset of spermatogenesis in fish. Germline Dev. (Series: Ciba Foundation Symposia), 182 (1994) 255–270.
[11] T. Miura, K. Yamauchi, Y. Nagahama, H. Takahashi, Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin, Zool. Sci. 8 (1991) 63–73.
[12] T. Miura, K. Yamauchi, H. Takahashi, H.Y. Nagahama, Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*), Proc. Natl. Acad. Sci. USA 88 (1991) 5774–5778.
[13] T. Kobayashi, H. Kajiura-Kobayashi, Y. Nagahama, A novel stage-specific antigen is expressed only in early stages of spermatogonia in Japanese eel, *Anguilla japonica* testis, Mol. Reprod. Dev. 51 (1998) 355–361.
[14] M. Yamashita, K. Mita, N. Yoshida, T. Kondo, Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects, Prog. Cell Cycle Res. 4 (2000) 115–129.
[15] H. Kajiura, M. Yamashita, Y. Katsu, Y. Nagahama, Isolation and characterization of goldfish cdc2, a catalytic component of maturation-promoting factor, Dev. Growth Differ. 35 (1993) 647–654.
[16] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[17] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.
[18] T. Kobayashi, H. Kajiura-Kobayashi, Y. Nagahama, Differential expression of *vasa* homologue gene in the germ cells during oogenesis and spermatogenesis in a teleost fish, tilapia, *Oreochromis niloticus*, Mech. Dev. 99 (2000) 139–142.
[19] M. Jackman, C. Lindon, E.A. Nigg, J. Pines, Active cyclin B1-Cdk1 first appears on centrosomes in prophase, Nat. Cell. Biol. 5 (2003) 143–148.
[20] F. Toyoshima, T. Moriguchi, A. Wada, M. Fukuda, E. Nishida, Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint, EMBO J. 17 (1998) 2728–2735.
[21] J. Li, A.N. Meyer, D.J. Donoghue, Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation, Proc. Natl. Acad. Sci. USA 94 (1997) 502–507.
[22] P. Gallant, E.A. Nigg, Cyclin B2 undergoes cell cycle-dependent nuclear translocation and, when expressed as a non-destructible mutant, causes mitotic arrest in HeLa cells, J. Cell Biol. 117 (1992) 213–224.
[23] J. Pines, T. Hunter, Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport, J. Cell Biol. 115 (1991) 1–17.
[24] K. Ookata, S. Hisanaga, T. Okano, K. Tachibana, T. Kishimoto, Relocation and distinct subcellular localization of p34cdc2–cyclin B complex at meiosis reinitiation in starfish oocytes, EMBO J. 11 (1992) 1763–1772.