BIOLOGY OF REPRODUCTION **65,** 1195–1200 (2001)

Nuclear Accumulation of Cyclin B1 in Mouse Two-Cell Embryos Is Controlled by the Activation of Cdc2[1]

Akihiro Ohashi, Naojiro Minami,[2] and Hiroshi Imai

Laboratory of Reproductive Physiology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

ABSTRACT

In the present study, the sequential expression and cellular localization of cyclin B1 was examined in two-cell mouse embryos to elucidate the mechanism of the two-cell block. One-cell embryos derived from in vitro fertilization were cultured with oviductal tissue (nonblocking condition) or without oviductal tissue (blocking condition) to establish the experimental conditions in which the embryos either overcome the two-cell block or do not. The amount of cyclin B1 gradually increased through the second cell cycle (through S to G_2 phase). However, the difference was not observed between culture conditions. This showed that even embryos exhibiting the two-cell block normally synthesize cyclin B1 through the cell cycle. Cyclin B1 in embryos cultured under nonblocking condition accumulates in the nucleus during the transition from the G_2 to the M phase, whereas that in embryos cultured in blocking condition localizes in the cytoplasm throughout the cell cycle. These data indicate that two-cell embryos cultured in blocking condition are able to normally synthesize cyclin B1 but have defects in nuclear accumulation of the protein. However, when two-cell blocked embryos were treated with okadaic acid, an activator of Cdc2 kinase, part of cyclin B1 in the embryos translocated into the nucleus. Moreover, treatment with butyrolactone I, a specific inhibitor of Cdc2 kinase, inhibits nuclear translocation of cyclin B1 in those embryos. These results suggest that Cdc2 kinase regulates the nuclear accumulation of cyclin B1 in mouse two-cell embryos.

cdc2, cyclin B1, mouse embryo, two-cell block

INTRODUCTION

In many mammalian species, the development of embryos, when they are cultured from the one-cell stage in vitro, is generally arrested at a specific developmental stage [1, 2]. In the mouse, embryos are generally arrested at the two-cell stage, a phenomenon that has been termed ''the two-cell block'' [3]. This phenomenon is also observed in hamster and rat embryos [2, 4].

To overcome the two-cell block, several culture systems have been developed. These include addition of the chelating agent ethylenediaminetetraacetic acid [5] or a potent scavenger of superoxide anion, superoxide dismutase [6, 7], to the culture medium and removal of phosphate [8] from the culture medium. In addition, the use of oviduct [9] or oviductal tissue [10] also contributes to in vitro development of mouse one-cell embryos. However, in some strains of the mouse, no culture system has been established to overcome the two-cell block [11]. Therefore, it is quite important to elucidate the mechanism of the two-cell block at the molecular level.

5-Bromo-2′-deoxy-uridine and thymidine are reported to be incorporated in two-cell blocked embryos [12, 13], and a microdensitometric analysis of DNA content has shown that two-cell blocked embryos complete DNA replication [3]. These results indicate that mouse two-cell blocked embryos are arrested at the G_2 phase specifically and have defects in the transition from the G_2 to the M phase.

In the eukaryotic cell cycle, initiation of mitosis requires the activity of M phase-promoting factor, a complex of a cyclin-dependent kinase Cdc2 and a B-type cyclin [14]. The level of cyclin B1 gradually increases from the late S to the G_2 phase, and it becomes associated with Cdc2. However, Wee1/Myt1 protein kinase keeps Cdc2/cyclin B1 kinase activity low [15, 16]. The Wee1 protein kinase families have been reported to negatively regulate Cdc2 kinase by phosphorylating the residues in its ATP-binding site, Tyr15 and Thr14. At the end of the G_2 phase, abrupt dephosphorylation of these sites by Cdc25C phosphatase triggers Cdc2 kinase activation, and as a result, the cell cycle can progress to the M phase [15, 17].

Furthermore, it has been demonstrated that Cdc2/cyclin B1 kinase activity is controlled not only by phosphorylation/dephosphorylation but also by its cellular localization. Pines et al. [18] and Ookata et al. [19] revealed that cyclin B1 localizes in the cytoplasm during the S and the G_2 phases. However, just before mitosis, a part of the cyclin B1 accumulates in the nucleus and localizes there until nuclear breakdown occurs at prometaphase [18, 19].

Cyclin B1 has a cytoplasmic-retention sequence (CRS) [20], which contains a hydrophobic, leucine-rich, nuclear-export sequence (NES) [21–23]. Furthermore, phosphorylation of four-amino acid residues near the NES region is associated with nuclear accumulation of cyclin B1 [24–26]. However, to our knowledge, nuclear translocation of cyclin B1 has not been investigated in mouse preimplantation embryos.

The Cdc2 kinase activity of mouse two-cell blocked embryos remains low [8, 13, 27], indicating that mouse two-cell embryos cultured in blocking condition have some defects in the control of Cdc2 kinase activation. In the present study, we examined the dynamics of cyclin B1 in mouse two-cell embryos using okadaic acid (OA), which activates Cdc2 kinase by inhibition of type 2A protein phosphatase [28–30], and using butyrolactone I (BL I), a specific inhibitor of Cdc2 kinase, to elucidate the two-cell block in terms of the cell cycle.

MATERIALS AND METHODS

In Vitro Fertilization and Embryo Culture

In vitro fertilization (IVF) and embryo culture were carried out as previously described [31]. Briefly, 3- to 4-wk-old female Crj:CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo, Japan) were superovulated with eCG and hCG. Ovulated oocytes were collected from the ampullae of the

[1]Part of this work was supported by a grant from the Ministry of Education, Science and Culture (no. 10660270 to N.M.) and a grant from the Japan Society for the Promotion of Science (JPS-RFTF 97L00905 to N.M.).
[2]Correspondence. FAX: 81 75 753 6329; e-mail: naojiro@kais.kyoto-u.ac.jp

Received: 27 February 2001.
First decision: 26 March 2001.
Accepted: 25 May 2001.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Developmental competence of mouse IVF embryos cultured with or without oviductal tissue.

Culture condition	No. of embryos cultured	No. (%) of embryos developed to	
		≤ 3-Cell stage	≥ 4-Cell stage
Coculture	116	6 (5.2)	110 (94.8)
Non-coculture	119	107 (89.9)	12 (10.1)

oviducts 16 h after hCG injection. Oviducts for coculture were flushed with modified Whitten medium (m-WM) containing 3 mg/ml of polyvinylpyrrolidone (PVP; K-30; Nacalai Tasque, Kyoto, Japan) at the time of oocyte collection, and then the isthmic and fimbrial regions were removed. The ampullae were dissected longitudinally to open the duct, washed several times with m-WM containing PVP, and then introduced into 100 μl of each culture medium (m-WM containing PVP, one ampulla per 100 μl). Spermatozoa were collected from the cauda epididymis of male mice of the same strain and cultured for 1 h in 400 μl of preincubation medium.

Five hours after insemination, the eggs were washed several times and then transferred to 100 μl of culture medium with an ampulla (nonblocking culture condition) or without an ampulla (blocking culture condition). Eggs were judged to be fertilized when formation of both the male and female pronuclei were visible at 5 h after insemination.

All incubations were performed in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

All animal experiments were performed with the approval of the Kyoto University Institutional Animal Care and Use Committee and in adherence with guidelines established in the Guide for Care and Use for Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

Embryo Collection

At 1, 4, 10, and 21 h after the first cleavage, which times correspond to the G1, S, early G_2, and late G_2 (G_2/M) phases of the second cell cycle of the embryos, respectively, embryos were collected for each analysis. In embryos cultured without oviductal tissue, the embryos in the two-cell stage at 28 h after the first cleavage were used for two-cell blocked embryos.

Immunoblotting

One-hundred embryos for the analysis of cyclin B1 or 30 embryos for the analysis of Cdc2 were collected at the end of each culture or treatment and then lysed in SDS sample buffer. The samples were boiled for 3 min to denature proteins and then stored at −80°C until use. Each sample was electrophoresed in a 12.0% (w/v) polyacrylamide gel containing SDS (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membranes were first incubated for 2 h in PBS-Tween (PBS-T; 136 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, and 0.1% [v/v] Tween-20) containing 10% (v/v) fetal calf serum (FCS). The blocked membranes were then incubated for 2 h with primary antibodies (1:250 dilution) against Cdc2 (Sc-54; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or cyclin B1 (Sc-752; Santa Cruz Biotechnology) in PBS-T containing 5% (v/v) FCS. After incubation with a biotinylated secondary antibody (1:1000 dilution) in PBS-T containing 10% (v/v) FCS for 1 h, the membranes were incubated with horse radish peroxidase-conjugated streptavidin (1:1000 dilution; Amersham Pharmacia Biotech, Buckinghamshire, England) for 1 h. The blots were detected using the enhanced chemiluminescence (Amersham Pharmacia Biotech) detection system according to the manufacturer's instructions.

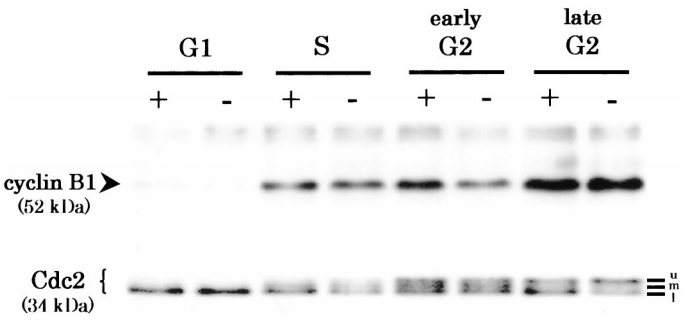

FIG. 1. Western blots of cyclin B1 and Cdc2 of mouse two-cell embryos. Upper panel shows cyclin B1 and lower panel Cdc2; + and − indicate embryos cultured under nonblocking and blocking condition, respectively. The G1, S, early G_2 and late G_2 (G_2/M) phases correspond to 1, 4, 10, and 21 h after the first cleavage, respectively. The letters u, m, and l indicate upper, middle, and lower bands of Cdc2, respectively. The amounts of cyclin B1 were not different between culture conditions at each cell-cycle stage (S: $P = 0.84$; early G_2: $P = 0.35$; late G_2: $P = 0.50$). The ratio of the upper band (inactive form) of Cdc2 at late G_2 between culture conditions was greater in blocking embryos than that in nonblocking embryos ($P = 0.04$). The experiment was repeated three times for cyclin B1 and four times for Cdc2. The results of one representative experiment are presented.

For positive control of cyclin B1, MII oocytes and fertilized eggs (3 h after fertilization) were used. Cyclin B1 protein was detected in MII oocytes, whereas the protein was not detected in fertilized eggs. For negative control, neither cyclin B1 nor Cdc2 proteins were detected in the absence of the primary antibodies. Molecular weights of Cdc2 and cyclin B1 were measured based on the molecular weight markers (Amersham Pharmacia Biotech). All incubations were performed at room temperature.

The intensities of the bands of cyclin B1 and Cdc2 were determined with a model 4.0 ATTO densitograph (ATTO, Inc., Tokyo, Japan). The amounts of cyclin B1 at each cell-cycle stage (S, early G_2, and late G_2) and the ratio of the upper band of Cdc2 at late G_2 phase in each culture condition (Fig. 1) was compared using Student *t*-test following ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC). A *P* value of less than 0.05 was considered to be significant.

Immunofluorescence

Embryos for immunocytochemistry were fixed in 3.7% paraformaldehyde in PBS for 1 h after removal of the zona pellucida with acid Tyrode solution (pH 2.5). After washing three times in PBS containing 1 mg/ml of BSA (PBS-BSA), fixed embryos were permeabilized for 1 h with PBS-PVP containing 0.1% (v/v) Triton X-100 and incubated for 60 min in 10% (v/v) goat normal serum in PBS (blocking solution). Following a 1-h incubation in PBS-BSA containing the primary antibody against cyclin B1 (Sc-752) at a dilution of 1:100, the embryos were washed in PBS-BSA and then incubated for 60 min in the dark in PBS-BSA containing Alexa 488 conjugated secondary antibody (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:200. Quantitation of fluorescence was carried out with a confocal laser microscope (Carl Zeiss, Oberkochen, Germany) and Zeiss image analysis system. For negative control, we performed the immunofluorescence experiment for cyclin B1 without primary antibody. All performances were carried out at room temperature.

Treatment of Embryos with OA

Okadaic acid (Sigma, St. Louis, MO), an inhibitor of type 2A and type 1 protein phosphatases, was used at a concentration of 2.5 μM in m-WM. Two-cell blocked embryos obtained from blocking condition were incubated in the medium containing OA for 1, 2, or 3 h.

Treatment of Embryos with BL I

Butyrolactone I (Calbiochem, San Diego, CA), a specific inhibitor of Cdc2, was used at a concentration of 100 μM in m-WM. The BL I was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Two-cell blocked embryos obtained from blocking condition were incubated in the medium containing OA and BL I for 3 h.

Chromosome Mitotic Index

The embryos treated with OA alone or with OA and BL I were fixed in 3.7% (w/v) paraformaldehyde in PBS for 1 h at room temperature. After washing in PBS, the fixed embryos were incubated for 30 min with 50 μg/ml of Hoechst 33258 (Sigma) at room temperature and examined with a fluorescent microscope (BX50; Olympus, Tokyo, Japan). Mitotic embryos were scored by their lack of nuclear membrane and evidence of chromosome condensation, and then the number of mitotic blastomeres was divided by the total number of blastomeres.

RESULTS

The first cleavage of most one-cell embryos, which formed both male and female pronuclei at 5 h after IVF,

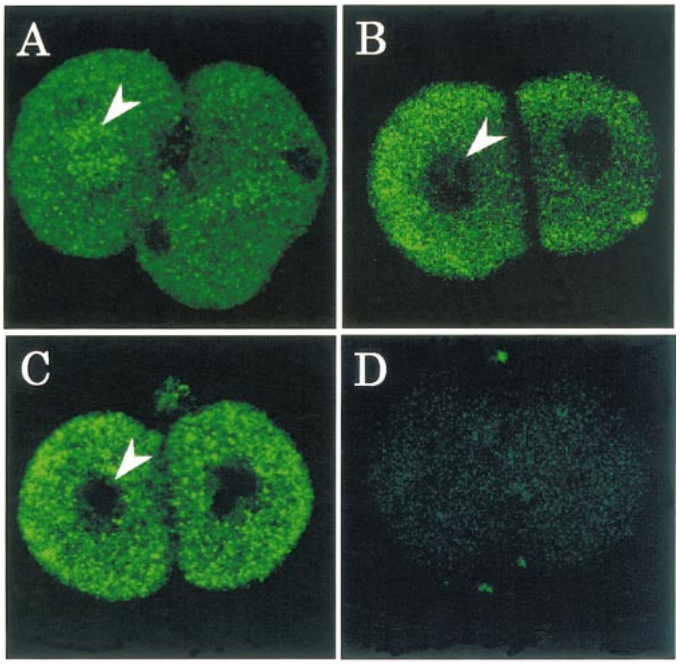

FIG. 2. Localization of cyclin B1 in mouse two-cell embryos cultured in nonblocking or blocking condition. Two-cell embryo at G_2/M phase cultured in nonblocking condition (**A**), two-cell embryo at late G_2 phase cultured in blocking condition (**B**), two-cell blocked embryo at 28 h after the first cleavage (**C**), and negative control without primary antibody (**D**) are shown. The G_2/M transition, late G_2 phase, and blocked correspond to 23–24, 21, and 28 h after the first cleavage, respectively. Arrowheads indicate the nucleus of embryos. The experiment was repeated three times, with 10–20 embryos in each replicate.

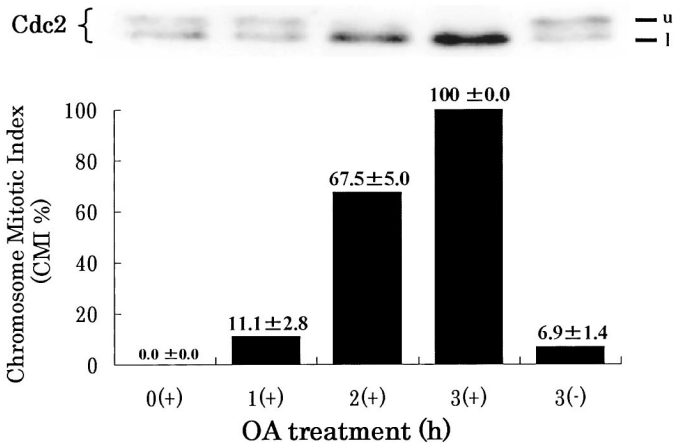

FIG. 3. Effect of OA on the activation of Cdc2 kinase and the G_2/M transition of mouse two-cell blocked embryos. Two-cell blocked embryos were incubated with (+) or without (−) 2.5 μM OA in m-WM for 0–3 h as described in *Materials and Methods*. Upper panel shows Western blot analysis of Cdc2 in 2-cell blocked embryos treated with or without OA, corresponding to the lower panel. The letters u and l indicate the upper and lower bands of Cdc2, respectively. Lower panel shows chromosome mitotic index in two-cell blocked embryos treated with (+) or without (−) OA. Data are presented as the mean ± SEM (n = 3).

was achieved up to 17–20 h after IVF. As for the second cleavage, 94.8% of two-cell embryos cultured in nonblocking condition developed beyond the four-cell stage, whereas 89.9% of two-cell embryos cultured in blocking condition could not develop to the four-cell stage (Table 1).

As shown in Figure 1, cyclin B1 protein appeared at the S phase in embryos cultured under both blocking and nonblocking conditions, and the amount of the protein seemed to be constant during the S and the early G_2 phase. However, the amount of the protein became more abundant at the late G_2 (G_2/M) phase in both blocking and nonblocking culture conditions. Moreover, at the late G_2 (G_2/M) phase, the amount of activated Cdc2 (lower band [8, 27]) became abundant in embryos cultured in nonblocking condition, whereas preactivated Cdc2 (upper band) accumulated in embryos cultured in blocking condition.

Immunofluorescence staining showed that, in some of the embryos cultured under nonblocking condition, part of the cyclin B1 accumulated in the nucleus at the beginning of the M phase, whereas in embryos cultured under blocking condition and in two-cell blocked embryos, cyclin B1 localized in the cytoplasm throughout the cell cycle (Fig. 2).

As shown in Figure 3, 67.5% of two-cell blocked embryos treated with OA for 2 h and 100% of embryos treated with OA for 3 h progressed to the M phase in accordance with the Cdc2 kinase activation. Immunostaining showed that, in some two-cell blocked embryos treated with OA for 1.5 h, part of the cyclin B1 accumulated in the nucleus,

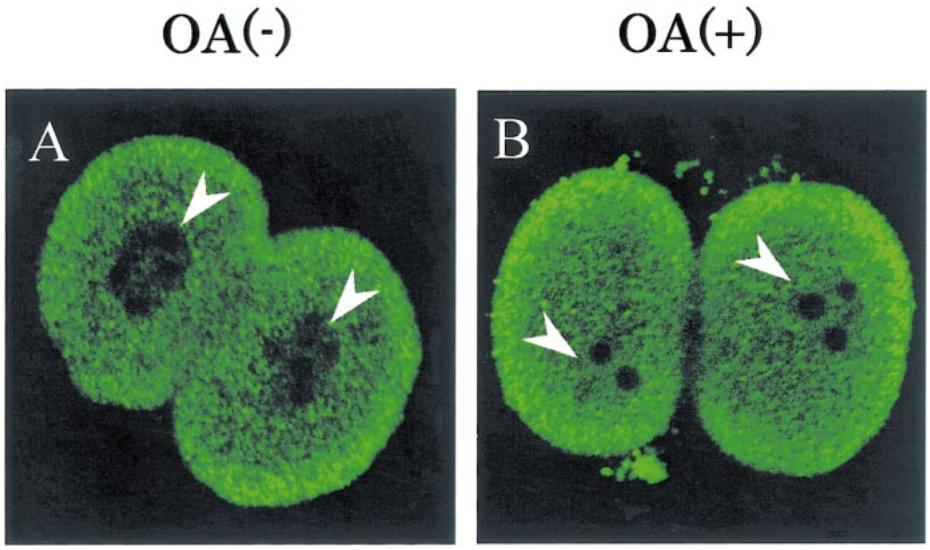

FIG. 4. Localization of cyclin B1 in mouse two-cell blocked embryos treated with or without OA. Two-cell blocked embryos were incubated without OA (**A**) or with (**B**) 2.5 μM OA in m-WM for 1.5 h as described in *Materials and Methods*. Arrowheads indicate the nucleus of embryos. The experiment was repeated three times, with 10–20 embryos in each replicate.

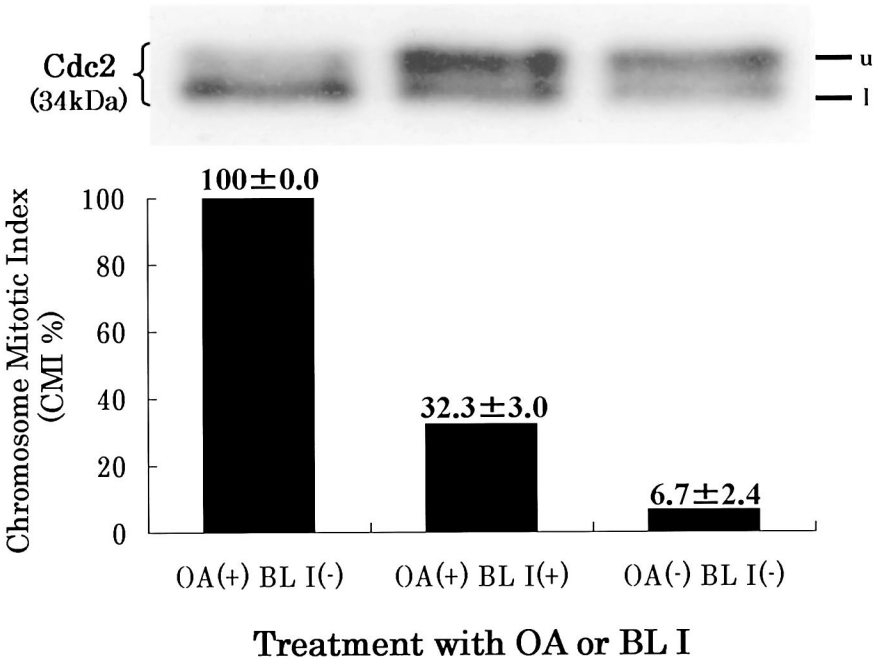

FIG. 5. Butyrolactone I inhibits the G_2/M transition through inactivation of Cdc2 kinase in mouse two-cell blocked embryos. Two-cell blocked embryos were incubated with 2.5 μM OA alone (OA(+), BL I(−)) or together with 100 μM BL I (OA(+), BL I(+)) in m-WM for 3 h as described in *Materials and Methods.* Upper panel shows Western blot analysis of Cdc2 in two-cell blocked embryos treated with OA alone or together with BL I, corresponding to the lower panel. The letters u and l indicate upper and lower bands of Cdc2, respectively. Lower panel shows chromosome mitotic index in two-cell blocked embryos treated with OA alone or together with BL I. Data are presented as the mean ± SEM (n = 2).

whereas in all embryos cultured without OA for 1.5 h, cyclin B1 remained in the cytoplasm (Fig. 4).

To clarify the effect of Cdc2 kinase on the nuclear translocation of cyclin B1 in two-cell embryos, two-cell blocked embryos were treated with OA and BL I. As shown in Figure 5, nuclear membrane breakdown and/or chromosome condensation was inhibited in two-cell blocked embryos treated with OA and BL I, whereas the arrested cell cycle progressed to the M phase in embryos treated with OA alone. Moreover, according to the Western blot analysis, the upper band became intensive when embryos were treated with both OA and BL I compared with that of embryos treated with OA alone. For immunostaining, we used G_2-arrested embryos, which exhibit neither breakdown of nuclear membrane nor chromosome condensation after the treatment. Immunostaining showed that cyclin B1 remained in the cytoplasm in the embryos treated with OA and BL I as well as in two-cell blocked embryos treated with 1% DMSO alone (Fig. 6).

DISCUSSION

Because cyclin B1 regulates Cdc2 kinase activity by binding to Cdc2 [15, 17], we assumed that depression of cyclin B1 synthesis causes low activity of Cdc2 kinase in embryos cultured in blocking condition. However, the embryos cultured in blocking condition contain as much cyclin B1 protein as those cultured in nonblocking condition (Fig. 1), indicating that two-cell blocked embryos have enough cyclin B1 protein for the transition from the G_2 to the M phase. Therefore, the low activity of Cdc2 kinase in the two-cell blocked embryos is caused not by the amount of cyclin B1 but, rather, by the defects in Cdc2 dephosphorylation.

Cyclin B1 has been shown to localize in the cytoplasm during the S and G_2 phases in starfish oocytes [19], HeLa cells [18], and *Xenopus* oocytes [32, 33]. However, just before mitosis, part of the cyclin B1 translocates to the nucleus and accumulates there until the nuclear envelope

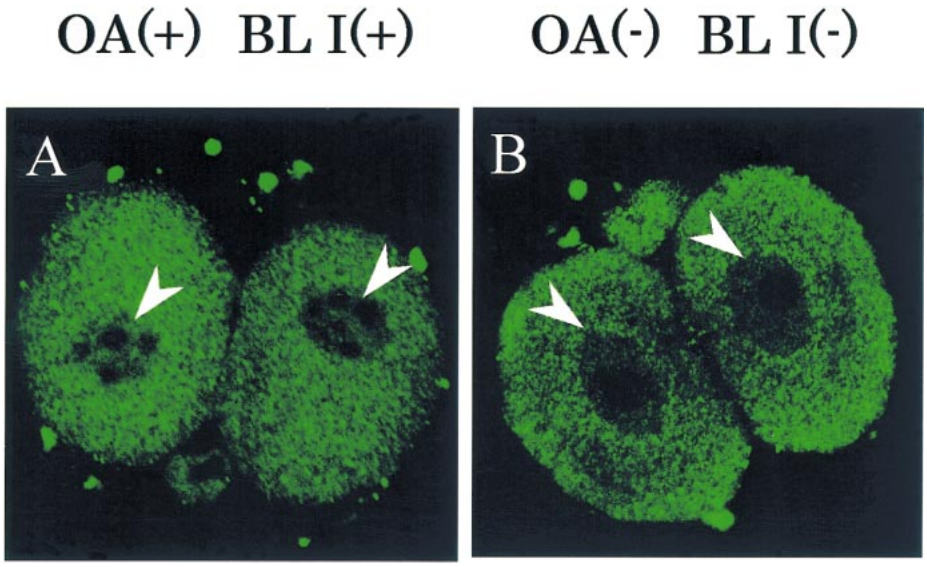

FIG. 6. Localization of cyclin B1 in mouse two-cell blocked embryos treated with OA and BL I. Two-cell blocked embryos were incubated with 2.5 μM OA and 100 μM BL I (**A**) or with 1% DMSO alone as control (**B**) in m-WM for 3 h as described in *Materials and Methods.* Arrowheads indicate the nucleus of embryos. The experiment was repeated three times, with 10–20 embryos in each replicate.

breaks down at prometaphase. Nevertheless, how nuclear translocation of cyclin B1 in mouse embryos is regulated remains unclear. In the present study, we showed that cyclin B1 in mouse two-cell embryos localizes in cytoplasm until the late G_2 phase, then suddenly accumulates in the nucleus at the G_2/M transition. On the other hand, cyclin B1 in two-cell blocked embryos localizes in the cytoplasm throughout the cell cycle, suggesting that the embryos exhibiting the two-cell block have some defects in the mechanism of nuclear translocation of cyclin B1.

Treatment of two-cell blocked mouse embryos with OA activates Cdc2 kinase and allows the arrested cell cycle to proceed to the M phase [8, 13, 27]. The specific inhibition of protein phosphatase 2A (PP2A) by OA induces the activation of Cdc25C phosphatase, which subsequently results in activation of Cdc2 kinase [29, 30, 34, 35]. Although how nuclear accumulation of cyclin B1 is induced in mouse embryos remains unclear, we showed in the present study that Cdc2 kinase activation by OA induces the accumulation of cyclin B1 in two-cell blocked embryos, and that this translocation of cyclin B1 is inhibited by BL I. It has been reported that BL I inhibits Cdc2 kinase selectively by competing with ATP [36, 37], so Cdc2 kinase activation may facilitate the nuclear accumulation of cyclin B1 by a positive feedback loop, in which activated Cdc2 kinase self-phosphorylates the CRS region of cyclin B1 [23]. The CRS region, which contains serine residues adjacent to a particular proline residue [38], can be the substrate for Cdc2 kinase [39]. However, cyclin B1 in *Xenopus* oocytes does not require Cdc2 for nuclear import [40], and cyclin B1 in DNA-damaged HeLa cells localizes in the cytoplasm even if Cdc2 kinase is activated [21]. Considering that the activation of Cdc2 kinase by OA induces cell-cycle progression from the G_2 to the M phase in mouse two-cell blocked embryos, it can be concluded that two-cell blocked embryos are not arrested in response to DNA damage at the G_2 phase. On the other hand, Cdc2 activity promotes nuclear accumulation of cyclin B1 in *Xenopus* oocytes [41], and nuclear localization of cyclin B1 in HeLa cells meditates Cdc2 kinase activity [33].

In the present study, we showed that part of the cyclin B1 localizes in the nucleus when Cdc2 kinase is activated in two-cell embryos cultured in nonblocking condition and in two-cell blocked embryos treated with OA. In addition, suppression of Cdc2 kinase activity by BL I inhibits nuclear translocation of cyclin B1 in the embryos treated with OA. Thus, the present study supports the hypothesis that the nuclear accumulation of cyclin B1 may be associated with Cdc2 kinase activity in mouse two-cell embryos.

In conclusion, embryos cultured in blocking condition are able to synthesize cyclin B1 protein normally. Furthermore, cyclin B1 in mouse two-cell blocked embryos localizes in cytoplasm, and Cdc2 kinase activation induces nuclear accumulation of cyclin B1 in two-cell embryos cultured in nonblocking condition or in two-cell blocked embryos treated with OA. These results reveal that nuclear translocation of cyclin B1 is important for the G_2/M transition, and that Cdc2 kinase controls the cellular localization of cyclin B1 in mouse two-cell embryos. Finally, mouse two-cell blocked embryos provide an excellent model for elucidating the mechanism of the cell cycle, especially the G_2/M transition, and to better understand the embryonic cell cycle.

REFERENCES

1. Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 1990; 26:90–100.
2. Bavister BD. Role of oviductal secretions in embryonic growth in vivo and in vitro. Theriogenology 1988; 29:143–154.
3. Goddard MJ, Pratt HP. Control of events during early cleavage of the mouse embryo: an analysis of the ''two-cell block.'' J Embryol Exp Morphol 1983; 73:111–133.
4. Mayer JFJ, Fritz HI. The culture of preimplantation rat embryos and the production of allophenic rats. J Reprod Fertil 1974; 39:1–9.
5. Abramczuk J, Solter D, Koprowski H. The beneficial effect EDTA on development of mouse one-cell embryos in chemically defined medium. Dev Biol 1977; 61:378–383.
6. Fissore RA, Jackson KV, Kiessling AA. Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. Biol Reprod 1989; 41:835–841.
7. Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T. Involvement of superoxide radicals in the mouse two-cell block. Mol Reprod Dev 1991; 28:356–360.
8. Haraguchi S, Naito K, Azuma S, Sato E, Nagahama Y, Yamashita M, Toyoda Y. Effects of phosphate on in vitro 2-cell block of AKR/N mouse embryos based on changes in cdc2 kinase activity and phosphorylation states. Biol Reprod 1996; 55:598–603.
9. Biggers JD, Gwatkin RLB, Bringster RL. Development of mouse embryos in organ culture of fallopian tubes on a chemically defined medium. Nature 1962; 194:747–749.
10. Minami N, Utsumi K, Iritani A. Effects of low molecular weight oviductal factors on the development of mouse one-cell embryos in vitro. J Reprod Fertil 1992; 96:735–745.
11. Suzuki O, Asano T, Yamamoto Y, Takano K, Koura M. Development in vitro of preimplantation embryos from 55 mouse strains. Reprod Fertil Dev 1996; 8:975–980.
12. Luthardt FW, Donahue RP. DNA synthesis in developing two-cell mouse embryos. Dev Biol 1975; 44:210–216.
13. Minami N, Kojima T, Ymada M, Utsumi K. Relationship between p34cdc2 kinase activity and developmental competence of two-cell mouse embryos cultured in an oviductal environment. Biol Reprod 1997; 56:139 (abstract).
14. Nurse P. Universal control mechanism regulating onset of M-phase. Nature 1990; 344:503–508.
15. King RW, Jackson PK, Kirschner MW. Mitosis in transition [see comments]. Cell 1994; 79:563–571.
16. De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH. Crystal structure of cyclin-dependent kinase 2. Nature 1993; 363: 595–602.
17. Lew DJ, Kornbluth S. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr Opin Cell Biol 1996; 8: 795–804.
18. Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J Cell Biol 1991; 115:1–17.
19. Ookata K, Hisanaga S, Okano T, Tachibana K, Kishimoto T. Relocation and distinct subcellular localization of p34cdc2-cyclin B complex at meiosis reinitiation in starfish oocytes. EMBO J 1992; 11:1763–1772.
20. Pines J, Hunter T. The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J 1994; 13:3772–3781.
21. Toyoshima F, Moriguchi T, Wada A, Fukuda M, Nishida E. Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G_2 checkpoint. EMBO J 1998; 17:2728–2735.
22. Hagting A, Karlsson C, Clute P, Jackman M, Pines J. MPF localization is controlled by nuclear export. EMBO J 1998; 17:4127–4138.
23. Yang J, Bardes ES, Moore JD, Brennan J, Powers MA, Kornbluth S. Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes Dev 1998; 12:2131–2143.
24. Fornerod M, Ohno M, Yoshida M, Mattaj IW. CRM1 is an export receptor for leucine-rich nuclear export signals [see comments]. Cell 1997; 90:1051–1060.
25. Ossareh-Nazari B, Bachelerie F, Dargemont C. Evidence for a role of CRM1 in signal-mediated nuclear protein export. Science 1997; 278: 141–144.
26. Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, Nishida E. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature 1997; 390:308–311.
27. Aoki F, Choi T, Mori M, Yamashita M, Nagahama Y, Kohmoto K. A deficiency in the mechanism for p34cdc2 protein kinase activation in mouse embryos arrested at two-cell stage. Dev Biol 1992; 154:66–72.
28. Rime H, Jessus C, Ozon R. Tyrosine phosphorylation of p34cdc2 is

regulated by protein phosphatase 2A in growing immature *Xenopus* oocytes. Exp Cell Res 1995; 219:29–38.
29. Hoffmann I, Clarke PR, Marcote MJ, Karsenti E, Draetta G. Phosphorylation and activation of human cdc25-C by cdc2–cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J 1993; 12:53–63.
30. Clarke PR, Hoffmann I, Draetta G, Karsenti E. Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in *Xenopus* egg extracts. Mol Biol Cell 1993; 4:397–411.
31. Minami N, Sasaki K, Aizawa A, Miyamoto M, Imai H. Analysis of gene expression in mouse two-cell embryos using fluorescein differential display: comparison of culture environments. Biol Reprod 2001; 64:30–35.
32. Li J, Meyer AN, Donoghue DJ. Requirement for phosphorylation of cyclin B1 for *Xenopus* oocyte maturation. Mol Biol Cell 1995; 6: 1111–1124.
33. Li J, Meyer AN, Donoghue DJ. Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. Proc Natl Acad Sci U S A 1997; 94:502–507.
34. Yamashita K, Yasuda H, Pines J, Yasumoto K, Nishitani H, Ohtsubo M, Hunter T, Sugimura T, Nishimoto T. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. EMBO J 1990; 9:4331–4338.
35. Kinoshita N, Yamano H, Niwa H, Yoshida T, Yanagida M. Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. Genes Dev 1993; 7:1059–1071.
36. Kitagawa M, Okabe T, Ogino H, Matsumoto H, Suzuki-Takahashi I, Kokubo T, Higashi H, Saitoh S, Taya Y, Yasuda H. Butyrolactone I, a selective inhibitor of cdk2 and cdc2 kinase. Oncogene 1993; 8: 2425–2432.
37. Kitagawa M, Higashi H, Takahashi IS, Okabe T, Ogino H, Taya Y, Hishimura S, Okuyama A. A cyclin-dependent kinase inhibitor, butyrolactone I, inhibits phosphorylation of RB protein and cell cycle progression. Oncogene 1994; 9:2549–2557.
38. Hagting A, Jackman M, Simpson K, Pines J. Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol 1999; 9:680–689.
39. Moreno S, Nurse P. Substrates for p34cdc2: in vivo veritas? Cell 1990; 61:549–551.
40. Moore JD, Yang J, Truant R, Kornbluth S. Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. J Cell Biol 1999; 144:213–224.
41. Takizawa CG, Weis K, Morgan DO. Ran-independent nuclear import of cyclin B1-Cdc2 by importing beta. Proc Natl Acad Sci U S A 1999; 96:7938–7943.