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Cyclin A2-CDK2 regulates embryonic gene activation in 1-cell mouse embryos

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Abstract

Recruitment of maternal mRNA in mice appears essential for embryonic gene activation (EGA) that is initiated in the 1-cell stage. The identity of which recruited mRNAs is responsible, however, is not known. We report here that recruitment of cyclin A2 mRNA may be critical for EGA. Cyclin A2 protein accumulates in pronuclei between 6 and 12 h after fertilization, the time when EGA is initiated. This cyclin A2 may be generated from maternally recruited cyclin A2 mRNA because its accumulation was inhibited by 3′-deoxyadenosine, which inhibits mRNA polyadenylation. When CDK2 activity or pronuclear accumulation of cyclin A2 was inhibited with CDK2 inhibitors or by microinjected siRNAs, respectively, DNA replication was not inhibited but the increase of transcriptional activity was prevented. In addition, microinjection of recombinant cyclin A2-CDK2 protein increased transcriptional activity. Cyclin A2-CDK2 is activated following egg activation, because an increase in phosphorylation of retinoblastoma protein was observed using antibodies that recognize site-specific phosphorylation catalyzed by this kinase and treatment with a CDK2 inhibitor or microinjection with cyclin A2 siRNAs prevented the increase in retinoblastoma protein phosphorylation. These results suggest that recruitment of maternal cyclin A2 mRNA following egg activation is linked to EGA.

Keywords: Cyclin A2; Cyclin-dependent kinase 2; Embryonic gene activation; 1-Cell mouse embryo; Phosphorylation of retinoblastoma protein

Introduction

Developing mouse oocytes are transcriptionally active, but a global decrease in transcription starts around mid-growth phase such that there is little transcription in the fully grown oocyte. Following maturation and arrest at metaphase II, fertilization triggers resumption of meiosis and activation of the embryonic genome. The timing of embryonic gene activation (EGA) is species-specific (Braude et al., 1988; Camous et al., 1986; Crosby et al., 1988; Hoffert et al., 1997; Kelk et al., 1994; Manes, 1973) and in mouse is initiated in the 1-cell embryo, several hours after pronuclear formation (Aoki et al., 1997; Bounial et al., 1995; Matsumoto et al., 1994; Ram and Schultz, 1993); the identity of the genes transcribed in the 1-cell embryo, however, is poorly defined. EGA is quite robust in the 2-cell embryo and is responsible for a major reprogramming of gene expression that likely provides the molecular foundation of transforming the highly differentiated oocyte into the totipotent blastomeres of the 2-cell embryo.

EGA differs from initiation of transcription in somatic cells following entry into interphase. In somatic cells, transcription ceases only for a short time during mitosis and is then rapidly established following entry into

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interphase with formation of a nucleus. In contrast, transcription is absent during the ~14 h that it takes oocytes to mature and arrest at metaphase II, a condition that can be maintained for several additional hours prior to fertilization. Only when pronuclei have formed (~5–7 h post-fertilization) and DNA replication has initiated is transcription detected, i.e., about 10 h post-fertilization. In addition, whereas cytokines frequently modulate the gene expression profile via signal transduction pathways that converge on the nucleus, the dramatic reprogramming of gene expression that accompanies EGA only requires egg activation.

Although successful execution of EGA is essential for further development, little is known about the molecular basis that regulates EGA, although 1-cell mouse embryos have some transcription factors and RNA polymerase II (RNAP II) activity. For instance, nuclear concentrations of the transcription factor Sp1 and the TATA-box binding protein TBP increase during 1-cell stage (Worrad et al., 1994). Inhibiting phosphorylation of RNAP II subunits with bisindolylmaleimide I (BIM) in 1-cell embryos is correlated with a decrease in transcription (Miyara et al., 2003). In addition, although a transcriptionally permissive state is established during the 1-cell stage, the factors responsible for transcription, e.g., RNAP II, are universally required for gene expression. What is lacking is identifying specific mechanisms for initiating EGA in 1-cell mouse embryos.

Cytoplasmic poly(A) tail elongation of maternal mRNA is tightly coupled with translation of mRNAs and is a central mechanism for controlling translation of mRNA during maturation of *Xenopus laevis* oocytes. Recruitment of maternal mRNAs also occurs during maturation and following fertilization of mouse oocytes (Barkoff et al., 2000; Paynton and Bachvarova, 1994; Tay et al., 2000). For example, following fertilization, spindlin and cyclin A2 mRNAs undergo poly(A) tail elongation and translation (Fuchimoto et al., 2001; Oh et al., 2000). Interestingly, treating 1-cell mouse embryos with 3′-deoxyadenosine, which functions as a chain terminator of poly(A) tail elongation of maternal mRNA, but not 3′-deoxyguanosine, decreases transcriptional activity in 1-cell embryos (Aoki et al., 2003). This result led to the hypothesis that recruitment of some maternal mRNAs following fertilization is essential for EGA. This finding is also consistent with the observation that protein synthesis is required for EGA (Aoki et al., 2003; Wang and Latham, 1997; Wang and Latham, 2000). The identity of the maternal mRNAs recruited and essential for EGA, however, remains unknown.

Recruitment of maternal cyclin A2 mRNA could be such an mRNA. The amount of cyclin A2 protein increases from 6 h to 12 h post-insemination, i.e., when EGA is first observed in the 1-cell embryo, and translation of cyclin A2 mRNA is inhibited by 3′-deoxyadenosine (Fuchimoto et al., 2001). In somatic cells, cyclin A2, as well as cyclins D and E, activate specific cyclin-dependent kinases that in turn phosphorylate retinoblastoma protein (pRB). This phosphorylation induces conformational changes of pRB and releases the transcription factor E2F from pRB complex. Free E2F then directs gene expression involved in S-phase entry and S-phase progression.

We report here that inhibiting cyclin A2-CDK2 kinase with either roscovitine or siRNAs directed towards maternal cyclin A2 mRNA inhibits EGA in 1-cell embryos, whereas over-expressing cyclin A2-CDK2 leads to an increase in transcription. Results of these experiments suggest that recruitment of maternal cyclin A2 mRNA following fertilization leads to CDK2 activation that in turn triggers EGA in the 1-cell mouse embryo.

Materials and methods

In vitro fertilization and embryo culture

Female ddY mice, 21–23 days of age and mature ICR male mice were purchased from SLC Japan Inc. (Shizuoka, Japan). Metaphase II-arrested eggs (MII eggs) were obtained following superovulation of female mice with 5 IU of pregnant mare's serum gonadotrophin (Teikoku zouki, Tokyo, Japan) followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG) (Teikoku zouki). MII eggs embedded in cumulus cell masses were isolated from the ampullae of oviducts 15.5 h post-hCG injection and kept in Whitten's medium (Whitten, 1971). Sperm obtained from the *cauda epididymis* of ICR male mice and incubated for 2 h in Whitten's medium in a humidified atmosphere of 5% CO_2/95% air at 38°C were used for fertilization in vitro. Six hours after insemination, fertilized eggs were identified as those containing a male and female pronucleus and washed three times prior to culture in glucose-free CZB medium (Chatot et al., 1989). When *zona pellucida*-free eggs were used, the *zona pellucida* was removed by a brief treatment in acidic Minimum Essential Medium-compatible buffer (Acidic-MEMCO) (Evans et al., 1995a,b).

Treatment of embryos with CDK2 inhibitors and other inhibitors

Four hours after insemination, the embryos were transferred to Whitten's medium containing 250 μg/ml of hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 5 min at 37°C to remove cumulus cells. After washing three times in glucose-free CZB medium, the embryos were transferred to glucose-free CZB medium containing 100 μM of butyrolactone I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 100 μM of roscovitine (Sigma-Aldrich Co.). The embryos were cultured until 12 h post insemination at which time they were used for either the in vitro transcription assay or immunocytochemistry. When embryos were treated with

aphidicolin (final concentration of 3 μg/ml, Sigma-Aldrich Co.), cycloheximide (10 μg/ml, Sigma-Aldrich Co.), 3′-deoxyadenosine (2 mM, Sigma Chemical Co., St. Louis, MO, USA), or 3′-deoxyguanosine (2 mM, Sigma Chemical Co.), they were collected 1 h after insemination and transferred to glucose-free CZB medium containing these chemicals.

In vitro transcription assay

In vitro transcriptional activity assays were conducted as previously described (Kim et al., 2002). Briefly, 1-cell mouse embryos, whose plasma membrane had been permeabilized with Triton X-100, were incubated with BrUTP. The amount of incorporated BrU was measured by immunostaining with a primary antibody recognizing BrU (Roche Diagnostics Co., Indianapolis, IN, USA) and an FITC-conjugated secondary antibody. The intensity of the fluorescence signal was quantified using NIH-Image software (National Institute of Health, Bethesda, MD, USA). The relative amount of fluorescence in both the male and female pronuclei was combined in each embryo.

Immunoblotting

Immunoblotting was performed as described previously (Fuchimoto et al., 2001). Briefly, two hundred MII eggs and 1-cell embryos collected 12 h after insemination were subjected to SDS-PAGE (10% gel) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The primary and secondary antibodies used for the detection of CDK2 were an anti-cdk2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; catalog number sc-163) and anti-rabbit Ig, horseradish peroxidase-linked antibody (Amersham Biosciences, Buckinghamshire, UK; catalog number NA934V), respectively.

Immunostaining

Eggs and 1-cell embryos were immunostained as described previously (Kim et al., 2002), except for the following changes. The *zona pellucida* was removed using acidic-MEMCO before fixation as previously described (Evans et al., 1995a,b). Primary antibodies used were as follows: pRB, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; catalog number sc-50; phosphorylated Ser780 of pRB, Cell Signaling Technology Inc., Beverly, MA, USA; catalog number 9307; phosphorylated Ser249/Thr252, Thr356, Ser807/811, Thr821/826 of pRB, Santa Cruz Biotechnology, Inc.; catalog numbers sc-16671, sc-16837, sc-16670, and sc-16669, respectively; TFIID (TBP) and cyclin A2, Santa Cruz Biotechnology, Inc.; catalog numbers sc-204 and sc-751, respectively. To prepare working solutions of the primary antibodies, each antibody was diluted 1:100 with PBS/BSA. The embryos were incubated with each antibody for 1 h at room temperature, except for anti-cyclin A2 antibody, in which the incubation was performed overnight at 4°C. The fluorescence of the secondary FITC-conjugated antibody was detected using a Carl Zeiss 510 laser-scanning confocal microscope. The intensity of fluorescence was quantified using NIH-Image as described above.

Detection of DNA synthesis

DNA synthesis was detected by 5-bromo-2′-deoxyuridine (BrdU) incorporation as previously described (Aoki and Schultz, 1999). The embryos were collected 9 h after insemination, transferred to glucose-free CZB medium containing 10 μM of BrdU (Roche Diagnostics Co.), and incubated for 1 h at 37°C in CO_2 incubator. After HCl treatment, the embryos were subjected to immunostaining with 2 μg/ml of anti-BrdU monoclonal antibody (Roche Diagnostics Co.) for 1 h at room temperature and processed for laser-scanning confocal microscopy as described above.

Microinjection of cyclin A2-CDK2 protein into 1-cell embryos

Eggs were collected 1 h after insemination in vitro and used for microinjection. Microinjection was performed with an inverted microscope (ECLIPSE TE300, Nikon Co., Tokyo, Japan) using a micromanipulator (Narishige Co., Ltd., Tokyo, Japan) and microinjector (IM300, Narishige Co., Ltd.). After washing three times in Whitten's medium, 10–20 pl of cyclin A2-CDK2 active protein (0.2 μg/μl) (Upstate Biotechnology, Lake Placid, NY, USA; catalog number 14-448) was injected using borosilicate glass capillaries (GC100 TF-10, Harvard Apparatus Ltd., Kent, UK); heat-inactivated protein was used as the control. The injected embryos were cultured in glucose-free CZB medium, collected 6 h and 10 h after insemination, and subjected to immunostaining or in vitro transcriptional activity assay.

Cloning of mouse cyclin A2

Total mRNA was isolated from two hundred MII eggs using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Isolated mRNAs were reverse-transcribed in a 21-μl reaction mixture containing 1 μl of oligo (dT) primer (500 mg/ml, GIBCO BRL, Boston, MA, USA), 4 μl of 5× reaction buffer, 2 μl of DTT (0.1 M), 1 μl of dNTP mixture (four nucleotides, each at a concentration of 10 mM, GIBCO BRL), 1 μl of RNasin (40 units/ml, Promega Co.), and 10 units of Super Script II RNase H-Reverse Transcriptase (GIBCO BRL) at 42°C for 50 min.

Amplification of cyclin A2 cDNA by PCR was carried out in a 25-μl reaction mixture containing 2.5 μl of × 10 PCR reaction buffer, 1.5 μl of 25 mM $MgCl_2$, 2.5 μl of 2.5

mM dNTP mixture, 50 pmol of sense and antisense-primers, 1.25 units of Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 2 μl of cDNA solution. The following primers were used: mouse cyclin A2 sense primer, 5′-CCG CGA TGC CGG GCA CCT-3′; mouse cyclin A2 antisense primer, 5′-TCA CAC ACT TAG TGT CTC TG-3′. The samples were heated at 95°C for 10 min and 40 cycles of PCR were performed as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. The amplified products were separated on 2% agarose gel electrophoresis and visualized by staining with ethidium bromide. The band of the anticipated size (1237 bp) was cut out, the cDNA fragments were eluted and subcloned into a pCR II vector (Invitrogen Co., Carlsbad, CA, USA). DNA sequences were determined by the dideoxy method using the DNA sequencing system of Applied Biosystems Japan Ltd., Tokyo.

Preparation of diced siRNA (d-siRNA)

Small interfering RNA (siRNA) was prepared by dicing mouse cyclin A2 double-strand RNA (dsRNA). A pCR II plasmid containing the whole coding region of cyclin A2 was used as a template for *Pfu* DNA polymerase (Promega Co.) and cyclin A2 sense/antisense primers. PCR conditions were as follows: Following an initial heat treatment at 95°C for 1 min, PCR was performed for 15 cycles consisting of a denaturation step at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The amplified cDNA product was separated on 2% agarose gel electrophoresis and visualized. The product of the appropriate size (1237 bp) was cut out, and the cDNA fragments were eluted. This PCR product was then amplified in a second PCR reaction using *Pfu* DNA polymerase and the following primers: cyclin A2 T7-sense primer, 5′-TAA TAC GAC TCA CTA TAG GTA CTC CGC GAT GCC GGG CAC CT-3′; cyclin A2 T7-antisense primer, 5′-TAA TAC GAC TCA CTA TAG GTA CTT CAC ACA CTT AGT GTC TCT G-3′. The second PCR was performed through 25 cycles using the same conditions as in the first PCR reaction.

Both the cyclin A2 sense RNA and antisense RNA were transcribed from the purified second PCR product. In vitro transcription was performed in a 20-μl reaction mixture containing 2 μl of 10× reaction buffer, 2 μl of 10 mM NTP mixture, 40 units of RNasin (Promega Co.), 1 μg of the PCR product, and 20 units of T7 RNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) for 1 h at 37°C. RNase-free DNase I (20 units, Roche Diagnostics GmbH) was added, the sample incubated 30 min at 37°C, and the reaction terminated by adding 2 μl of 0.2 M EDTA. The single-strand RNAs (ssRNAs) were harvested by ethanol precipitation, resuspended, and 60 μg of ssRNAs was added to an RNase-free microcentrifuge tube. The tube was transferred to 250 ml of boiled water and left for 1–1.5 h to allow re-annealing and formation of dsRNA, which was "diced" using the BLOCK-iT™ Dicer RNAi Kits (Invitrogen Co.). The dsRNA (60 μg) was diced to siRNA in a 300-μl reaction mixture containing reaction buffer and 60 units of dicer enzyme for 16 h at 37°C. siRNA production was confirmed following electrophoresis in a 4% agarose gel stained with ethidium bromide and containing 10-bp DNA step ladder markers (Promega Co.). The diced siRNAs (d-siRNA) were then purified using the BLOCK-iT™ RNAi purification reagents and spin cartridges were supplied with BLOCK-iT™ kit (Invitrogen Co.). The purified d-siRNA was injected to MII eggs as described below.

d-siRNA against rabbit alpha-globin was prepared as a control. The cDNA of rabbit alpha globin was obtained by reverse transcription of the mRNA (Sigma Chemical Co.) and amplified with *Pfu* DNA polymerase (Promega Co.) using the following primers: globin sense primer, 5′-CCC GCT GAC AAG ACC AAC AT-3′ and globin antisense primer, 5′-CGA TAT TTG GAG GTC AGC AC-3′. PCR was performed for 15 cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. The band of the expected size (413 bp) was excised, and the cDNA fragments were subjected to another round of PCR (25 cycles) using *Pfu* DNA polymerase and following primers: Globin T7-sense primer, 5′-TAA TAC GAC TCA CTA TAG GTA CTC CCG CTG ACA AGA CCA ACA T-3′; Globin T7-antisense primer, 5′-TAA TAC GAC TCA CTA TAG GTA CTC GAT ATT TGG AGG TCA GCA C-3′. In vitro transcription and dicing reaction were performed as for cyclin A2.

Microinjection of d-siRNA into MII stage oocytes

MII eggs 15.5 h post-hCG injection were used for microinjection. Microinjection was performed essentially as described above except that the micropipette was held with a microelectrode holder (World Precision Instruments, Inc., Sarasota, FL, USA; catalog number MEH7W10) that is designed to provide an electrical coupling between d-siRNA solution-filled glass pipettes and high input impedance microelectrode amplifiers (Nihon Kohden Co., Tokyo, Japan; catalog number MEZ-8301). High input impedance induced the penetration of leading edge of capillary into *zona pellucida* and cell membrane and resulted in a high incidence of survival and fertilization: more than 60% of microinjected oocytes formed a pronucleus 6 h after insemination (511/787; 64.9%).

Metabolic labeling of the embryos with [^{35}S] methionine

Embryos were collected 10 h after insemination, transferred to CZB medium containing 1 mCi/ml of [^{35}S] methionine (Amersham Pharmacia Biotech UK

Limited, Buckinghamshire, England), and metabolically radiolabeled for 2 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. Acid insoluble radioactivity was then measured as previously described (Fuchimoto et al., 2001).

Statistical analysis

Statistical analysis was performed using Student's *t* test with a *P* value of <0.01 as being significant.

Results

Effects of 3′-deoxyadenosine and cycloheximide on pronuclear accumulation of cyclin A2

We previously reported that although cyclin A2 protein is not detected in eggs, it is detected following fertilization. Moreover, the increase in amount that occurs following fertilization is inhibited by 3′-deoxyadenosine (3-dA), an inhibitor of mRNA polyadenylation (Fuchimoto et al., 2001). What was not determined was its localization, which is important because cyclin A2 functions in the nucleus (Jackman et al., 2002; Pines and Hunter, 1991). EGA initiates at mid-S phase (Aoki et al., 1997) and hence we used immunostaining to localize cyclin A2 in 1-cell embryos collected 6 and 12 h post-insemination when the embryos are in G1 and S/G2, respectively (Fig. 1; Aoki et al., 1992; Aoki and Schultz, 1999). We also confirmed that no BrUTP incorporation was detected at 6 h, detected in a very small fraction of the embryos labeled at 8 h, and in all of the embryos labeled at 12 h (data not shown). Immunostaining showed that the amount of cyclin A2 was clearly enriched in the pronuclei, and increased between 6 and 12 h following fertilization (Fig. 2).

To ascertain whether nuclear accumulation of cyclin A2 was due to recruitment of maternal cyclin A2 mRNA, 1-cell embryos were cultured in the presence of 3-dA or cycloheximide (CHX). Both treatments inhibited pronuclear accumulation (Fig. 2). The 3-dA effect was specific because the effect of 3′-deoxyguanosine (3-dG) treatment was substantially less than that observed with 3-dA. The inhibitory effect of 3-dA accumulation is consistent with previous results that employed immunoblotting (Fuchimoto et al., 2001) and support the hypothesis that maternal cyclin A2 mRNA is polyadenylated and translated after fertilization. In somatic cells, newly synthesized cyclin A2 can bind to existing CDK2, and formation of the heterodimer permits nuclear accumulation (Jackman et al., 2002; Maridor et al., 1993). Results of immunoblotting experiments revealed that CDK2 is already present in the egg and that its mass doesn't increase following fertilization (Supplemental Fig. 1). Therefore, like somatic cells, the newly synthesized cyclin A2 may initiate nuclear accumulation of the cyclin A2-CDK2 complex (Jackman et al., 2002; Maridor et al., 1993).

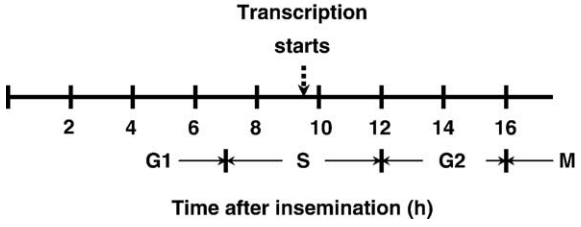

Fig. 1. Timing of cell cycle events in the 1-cell mouse embryo.

Effects of CDK2 inhibitors and the microinjection of cyclin A2 diced siRNA or cyclin A2-CDK2 protein on EGA

To examine whether the cyclin A2-CDK2 complex is involved in regulating EGA, 1-cell embryos were cultured in the presence of roscovitine (Ros) or butyrolactone I (BL1), potent CDK inhibitors. The presence of these inhibitors resulted in a marked decrease in transcriptional activity when assayed 12 h post-insemination (Fig. 3). These results suggested that cyclin A2-CDK2 regulates initiation of EGA. Note that fertilization triggers a rapid and dramatic decrease in cyclin B-CDK1 activity, which is essentially negligible 12 h post-insemination (Choi et al., 1991). Thus, it was unlikely that the observed effects of these inhibitors could be attributed to inhibiting CDK1. It was also unlikely that the effects were due to the inhibition of CDK4 or CDK6, because their expression levels markedly decreased to basal levels 6 h after fertilization (Kohoutek et al., 2004). Nevertheless, it was formally possible that another kinase inhibited by Ros or BL1 was responsible for initiating EGA. Therefore, we took an alternative approach to inhibit cyclin A2-CDK2 activity.

We elected to target maternal cyclin A2 mRNA for destruction prior to its recruitment following fertilization by an siRNA approach. The advantage of this method is that, by preventing fertilization-induced cyclin A2 protein synthesis, activation of CDK2 would be specifically prevented. RNAi functions in mouse oocytes and preimplantation embryos (Svoboda et al., 2001, 2004; Wianny and Zernicka-Goetz, 1999). Inhibition of CDK2 activation by cyclin A2 siRNAs was likely very specific, because it was unlikely that cyclin A2 siRNAs targeted transcripts of the other members of cyclin family, cyclin A1, E1, E2 and J, which can associate with CDK2 (Kolonin and Finley, 2000; Lauper et al., 1998; Sweeney et al., 1996). The analysis for nucleotide sequence of cyclin A2 cording region, which was used to prepare the siRNAs, revealed that there was no set of 21 consecutive nucleotides that are identical between cyclin A2 and the other cyclin family members. Furthermore, the highest matching was 19 of 21 nucleotides, which was found only in a single site of cyclin A1 sequence. In other cyclins, we did not find any

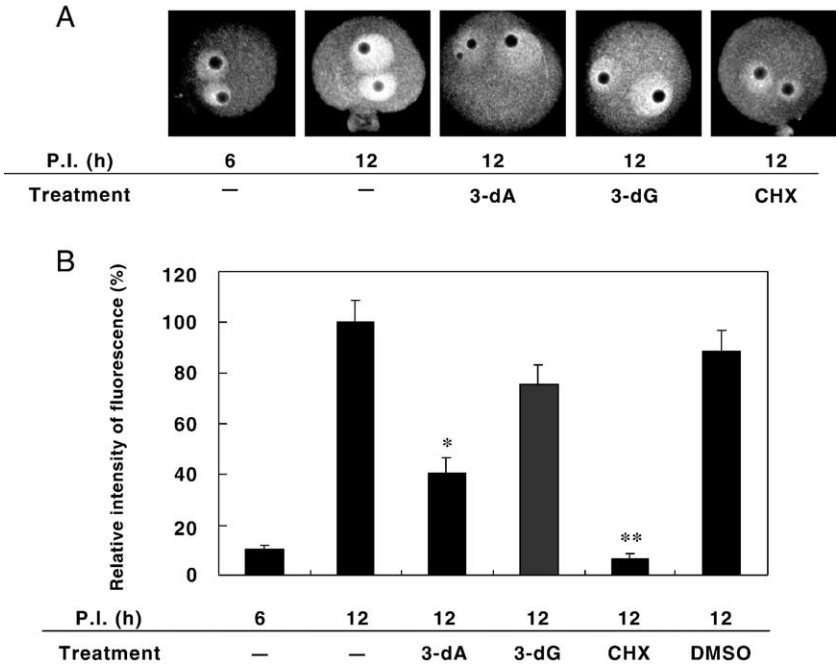

Fig. 2. Changes of pronuclear concentration of cyclin A2 during 1-cell stage and the effects of the treatment with 3′-deoxyadenosine (3-dA) and cycloheximide (CHX). (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-cyclin A2 antibody. One-cell embryos treated with 3-dA, 3′-deoxyguanosine (3-dG) and CHX were collected 12 h post-insemination (P.I.) and immunostained with anti-cyclin A2 antibody. (B) Relative fluorescence with an anti-cyclin A2 antibody in the embryos treated with 3-dA and CHX. The average value in the control embryos (12 h, non-treatment) was set as 100%. The experiment was conducted twice, and similar results were obtained in each case. The numbers of embryo examined were: 32, 29, 19, 27, 15, and 14 for the control embryos at 6 h, 12 h, and the embryos treated with 3-dA, 3-dG, DMSO, and CHX, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the value of the 3-dG (Student's t test, $P < 0.01$). Double asterisks (**) indicate significant difference from the value of the DMSO (Student's t test, $P < 0.01$).

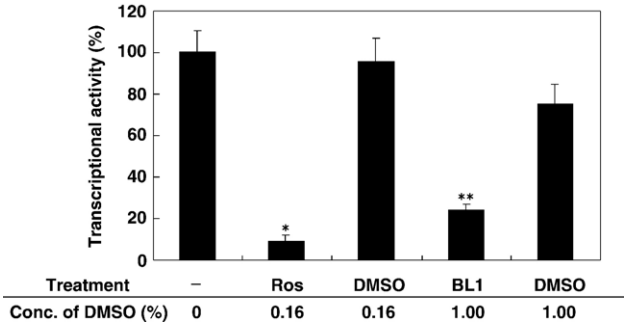

Fig. 3. Effects of roscovitine (Ros) and butyrolactone I (BL1) on BrUTP incorporation by the mouse 1-cell embryos. The embryos were collected 4 h after insemination and transferred to the culture medium containing Ros or BL1. DMSO was used as the solvent for Ros and BL1 and the final concentrations of DMSO in the culture medium were 0.16% and 1.00% for Ros and BL1, respectively. DMSO and CDK2 inhibitors were not added in culture medium for the control embryos (treatment minus). The embryos were collected 12 h after insemination and subjected to in vitro transcriptional activity assay. The average value in the control embryos was set as 100%. The experiments for Ros and BL1 were conducted three times and twice, respectively. Similar results were obtained in each case. The total numbers of embryo examined were: 39, 33, 37, 21, and 26 for the control, Ros, 0.16% DMSO, BL1, and 1% DMSO, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the value of the 0.16% DMSO (Student's t test, $P < 0.01$). Double asterisks (**) indicate significant difference from the value of the 1.00% DMSO (Student's t test, $P < 0.01$).

21 consecutive nucleotides in which more than 17 nucleotides were matched with those in cyclin A2. Furthermore, we injected siRNAs rather than dsRNA because it takes several hours for the dsRNA to be processed to siRNAs and in our experiment, a relatively rapid effect of RNAi-mediated mRNA destruction is required because de novo synthesis of cyclin A2 protein initiates as early as 6 h after insemination (Figs. 1–4; Fuchimoto et al., 2001).

Accordingly, we microinjected diced small interference RNA (d-siRNA), which had been prepared by dicing the full length of double-strand cyclin A2 mRNA into the MII eggs; the eggs then inseminated, and 12 h post-insemination, pronuclear cyclin A2 accumulation and transcriptional activity were examined. Immunostaining revealed that microinjection of cyclin A2 d-siRNA markedly decreased accumulation of cyclin A2 in the pronuclei (Figs. 4A, B). This effect was specific, because no effect was observed in accumulation of the TATA binding protein (TBP), whose concentration in the pronucleus increases after fertilization (Worrad et al., 1994). Furthermore, microinjection of rabbit globin d-siRNA did not affect the nuclear accumulation of cyclin A2. Last, these d-siRNA had little effect on the total protein synthesis, which was assessed by the incorporation of [^{35}S]methionine into TCA-precipitated macromolecule (Supplemental Fig. 2). Coupled with this decrease in

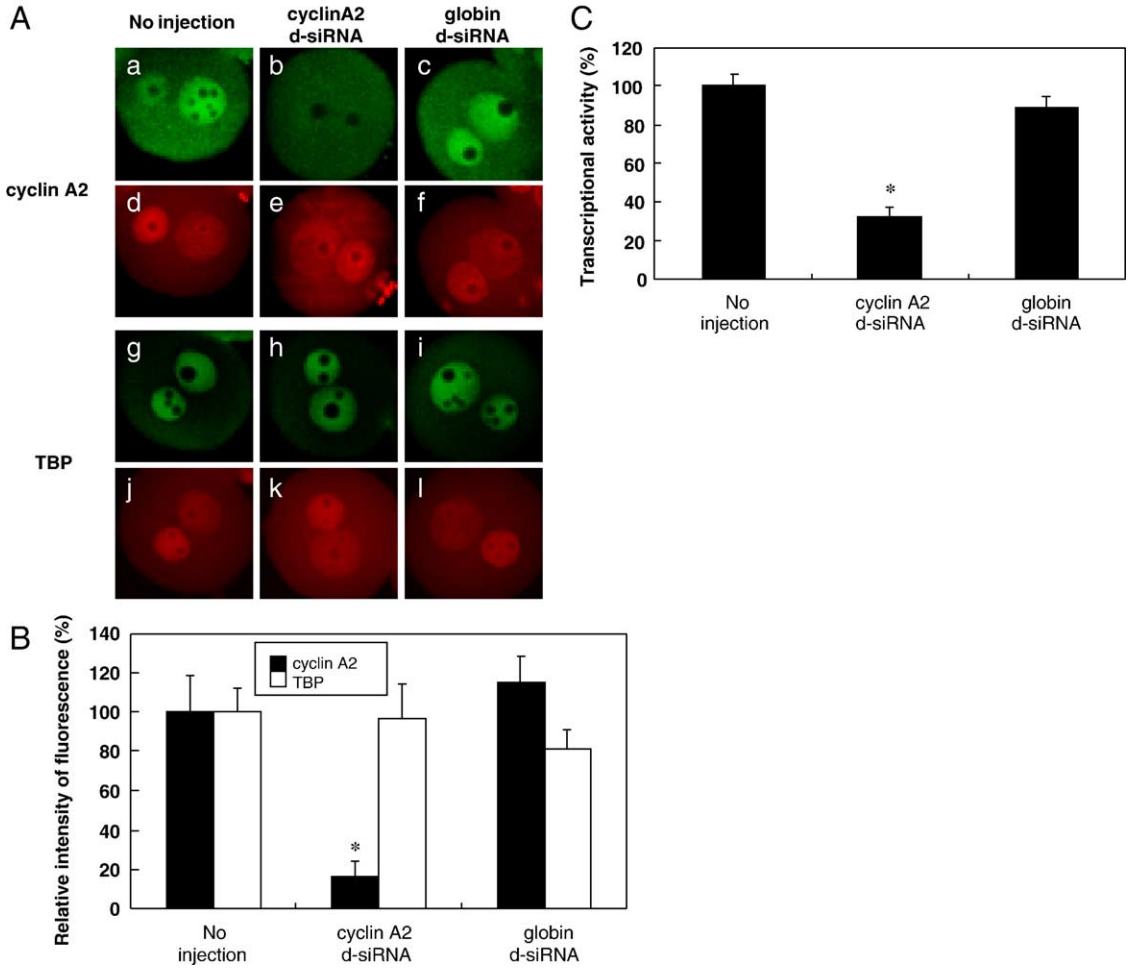

Fig. 4. Inhibition of cyclin A2 accumulation in the nucleus and transcriptional activity by cyclin A2 diced siRNA (d-siRNA). (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-cyclin A2 antibody (a–c) and anti-TBP antibody (g–i). DNA was stained with propidium iodide (d–f, j–l). MII oocytes were microinjected with cyclin A2 d-siRNA (b, e, h, k) or globin d-siRNA (c, f, i, l) and collected 12 h after insemination and immunostained. (B) The relative intensity of pronuclear fluorescence in the embryos injected d-siRNA. The intensity of fluorescence was measured in the embryos after immunostaining with anti-cyclin A2 and anti-TBP antibodies. The average value in the control embryos with no injection was set as 100%. The microinjection experiments were perform three times in which the embryos were immunostained with anti-cyclin A2 antibody and twice with anti-TBP antibody. Similar results were obtained in each case. The total numbers of embryo examined were: 11, 10, 17, 8, 14 and 11 for control/cyclin A2 immunostain, control/TBP immunostain, globin d-siRNA injection/cyclin A2 immunostain, globin d-siRNA injection/TBP immunostain, cyclin A2 d-siRNA injection/cyclin A2 immunostain and cyclin A2 d-siRNA injection/TBP immunostain, respectively. The columns and bars represent means ± SEM. An asterisk (*) indicates significant difference from the values of the no injection and globin d-siRNA injection (Student's *t* test, $P < 0.01$). (C) Effects of the microinjection of cyclin A2 d-siRNA on transcriptional activity in the mouse 1-cell embryos. MII oocytes were microinjected with cyclin A2 d-siRNA or globin d-siRNA, collected 12 h after insemination and subjected to in vitro transcriptional activity assay. The average value in noninjected embryos was set as 100%. The experiments were conducted three times. Similar results were obtained in each case. The total numbers of embryos examined were 16, 38, and 35, for no injection, cyclin A2 d-siRNA injection, and globin d-siRNA injection, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the values of the no injection and globin d-siRNA injection (Student's *t* test, $P < 0.01$).

pronuclear cyclin A2 was a significant reduction ($P < 0.01$) in transcriptional activity in embryos microinjected with cyclin A2 d-siRNA when compared to noninjected embryos or embryos injected with rabbit globin d-siRNA (Fig. 4C). These results strongly suggest that cyclin A2 regulates EGA in the 1-cell embryos, presumably via CDK2, which is cyclin A2's only known CDK binding partner during interphase (Sherr, 1996).

Cyclin A2 is essential for progression of S phase in the somatic cells. It was therefore possible that the reduction of cyclin A2-CDK2 activity by the CDK inhibitors or microinjection of cyclin A2 d-siRNA either inhibited entry into S phase or retarded phase progression, which would lead to reducing transcription in 1-cell embryos. To address this possibility, we analyzed the effects of CDK inhibitors and cyclin A2 d-siRNA on the DNA synthesis in 1-cell embryos. As shown in Table 1, DNA synthesis was not inhibited by these treatments; incorporation of BrdU was detected in all embryos subjected to these treatments, although no incorporation was observed in the embryos treated with aphidicolin. Furthermore, no difference in the intensity of the BrdU signal was observed in embryos in any experimental group.

Table 1
Effects of CDK2 inhibitors and the microinjection of diced siRNA on DNA synthesis in the mouse 1-cell embryos

Treatment	No. of experiment	No. of embryos		
		Total analyzed	BrdU signal	
			+	–
No treatment	3	125	125	0
DMSO[a]	3	85	85	0
Roscovitine[b]	3	65	65	0
Butyrolactone I[b]	3	69	69	0
Globin d-siRNA	2	21	21	0
Cyclin A2 d-siRNA	2	15	15	0
Aphidicolin[c]	2	12	0	12

[a] DMSO was added at a concentration of 0.16% or 1.00%, because it was used as the solvent for roscovitine (Ros) and butyrolactone I (BL1) and the final concentrations of DMSO were 0.16% and 1.00% for Ros and BL1, respectively.
[b] The concentration of Ros and BL1 was 100 μM.
[c] The concentration of aphidicolin was 3 μg/ml.

Nevertheless, cleavage to the 2-cell stage was inhibited in these embryos. One-third of the fertilized eggs that had been microinjected with cyclin A2 d-siRNA remained at the 1-cell stage 24 h after insemination, whereas more than 90% of the fertilized eggs that had been microinjected with rabbit globin d-siRNA cleaved to the 2-cell stage (data not shown). This inhibition likely reflects the fact that cyclin A2 functions at G2/M transition, as well as during S phase (Pagano et al., 1992; Loyer et al., 1994), i.e., during 1-cell stage, cyclin A2 functions as a cell cycle regulator at G2/M transition but not at S phase. These results demonstrated that cyclin A2-CDK2 appears to regulate EGA by a mechanism independent of the cell cycle.

To confirm further a role for cyclin A2-CDK2 in regulating EGA, we microinjected recombinant cyclin A2-CDK2 protein into 1-cell embryos 1 h after insemination. The embryos were then cultured, and 10 h after insemination, they were processed for in vitro transcriptional activity assay. Results of these experiments show that injection of active cyclin A2-CDK2 protein increased transcriptional activity by ~3-fold relative to embryos injected with heat-inactivated cyclin A2-CDK2 (Fig. 5).

Effects of CDK2 inhibitors and the microinjection of cyclin A2 diced siRNA on phosphorylation of pRB in the mouse 1-cell embryos

The tacit assumption underlying interpreting the results of the experiments described above is that cyclin A2-CDK2 is active in the 1-cell embryo during the time of EGA. To address this question, we assessed the phosphorylation state of pRB using antibodies specific for pRB forms that are preferentially phosphorylated by cyclin A2-CDK2 and the effect of inhibiting this kinase on the state of pRB phosphorylation.

pRB is phosphorylated on several sites of which Ser249/Thr252, Thr356, Ser807/811, and Thr821/826 are preferentially phosphorylated by cyclin A2-CDK2 (Kitagawa et al., 1996). Consistent with the increase in nuclear concentration of cyclin A2 and an increase in cyclin A2-CDK2 kinase activity following fertilization, phosphorylation of these sites significantly increased between 6 and 12 h post-insemination (Figs. 6A, B). The total amount of pRB, which was assayed with an antibody that recognizes both phosphorylated and unphosphorylated pRB, was not changed. Nevertheless, phosphorylation of Ser780, which is the site phosphorylated by cyclin D/cdk4 but not cyclin A2-CDK2 (Kitagawa et al., 1996), was not detected in the 1-cell embryos (Fig. 6C). Following cleavage to the 2-cell stage, a faint signal was detected in the nucleus at the early 2-cell stage and the signal intensity was significantly higher by the late 2-cell stage.

To provide additional evidence that the increase in pRB phosphorylation during 1-cell stage was mediated by CDK2, 1-cell embryos were cultured in the presence of roscovitine. Results of these experiments demonstrated that phosphorylation levels of Ser249/Thr252, Thr356, Ser807/811, and Thr821/826 were significantly decreased ($P < 0.01$), but that total amount of pRB was not changed (Figs. 6A, B). These results suggested that cyclin A2-CDK2 catalyzed pRB as a substrate around the time of EGA in the 1-cell embryos. Nevertheless, it was formally possible that Ros inhibited another CDK or protein kinase that was responsible for pRB phosphorylation at these sites. In addition, the contribution of cyclin A2 synthesized by recruitment of maternal cyclin A2 mRNA following fertilization was not addressed. Accordingly, we inhibited CDK2 activity by preventing accumulation of newly synthesized cyclin A2 protein by either blocking protein synthesis, or more specifically destroying cyclin mRNA by siRNAs.

Inhibiting protein synthesis with cycloheximide decreased phosphorylation of pRB on Ser249/Thr252, Thr356, and

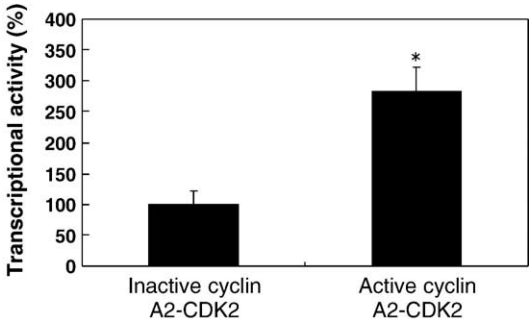

Fig. 5. Effects of cyclin A2-CDK2 protein injection on BrUTP incorporation in the mouse 1-cell embryos. One-cell embryos were microinjected with active or heat-inactivated cyclin A2-CDK2 protein, collected 10 h after insemination and subjected to in vitro transcriptional activity assay. The average value in the embryos microinjected with inactive cyclin A2-CDK2 protein was set as 100%. The experiments were conducted three times. Similar results were obtained in each case. The total numbers of embryo examined were 34 and 29 for inactive protein injection and active protein injection, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the value of the inactive cyclin A2-CDK2 injection (Student's t test, $P < 0.01$).

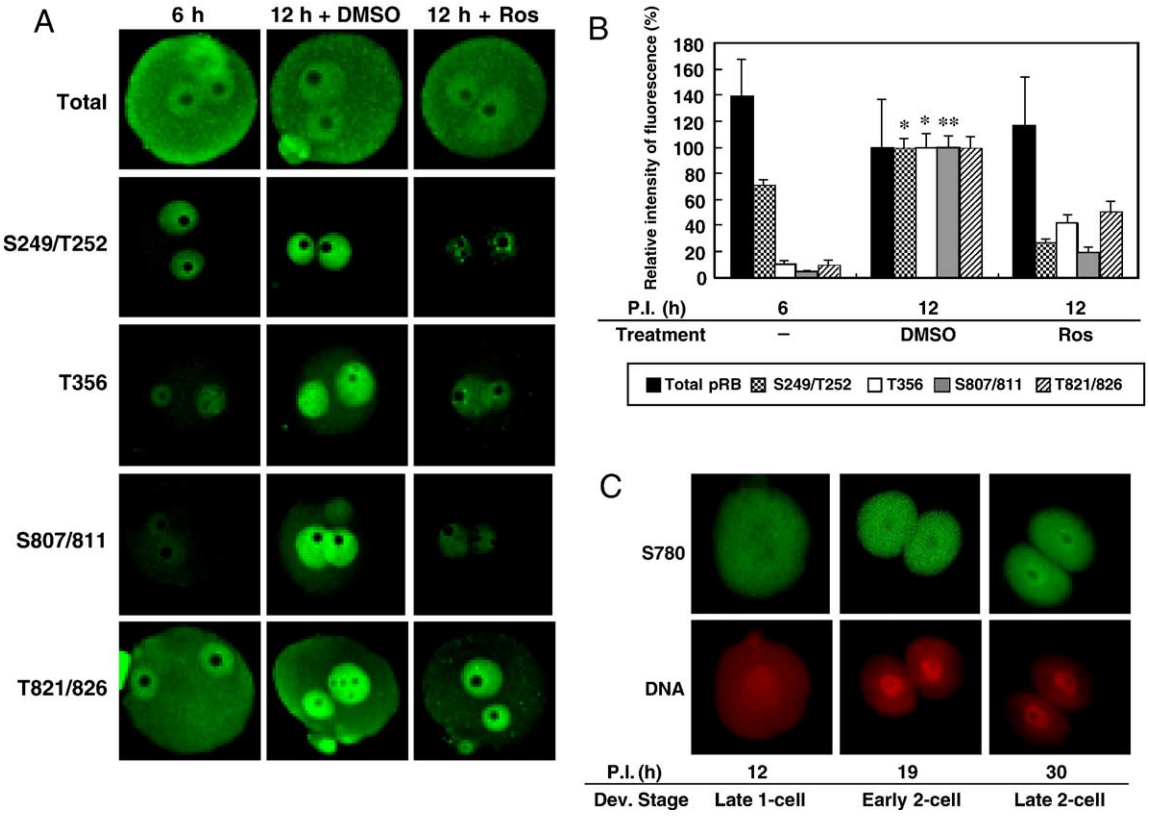

Fig. 6. Phosphorylation of pRB by CDK2 during 1-cell stage. The embryos were incubated with the CZB medium containing roscovitine (Ros) or 0.15% DMSO. The embryos were collected 6 and 12 h post-insemination (P.I.) and immunostained with the antibodies against pRB (Total), phospho Ser249/Thr252-pRB (S249/T252), phospho Thr356-pRB (T356), phospho Ser807/811-pRB (S807/811), and phospho Thr821/826-pRB (T821/826). (A) Confocal microscopic images of the mouse 1-cell embryos immunostained with anti-pRB antibody or site-specific anti-phospho pRB antibodies. (B) The relative intensities of pronuclear fluorescence in the embryos immunostained with anti-pRB antibody and site-specific anti-phospho pRB antibodies. The average value in the embryos treated with DMSO was set as 100%. The experiments for phospho S249/T252, T356, and S807/811-pRB were conducted three times, and those for phospho T821/826 and total pRB were twice. Similar results were obtained in each case. Eight or more embryos were analyzed in any experimental groups. The columns and bars represent mean ± SEM. Asterisks (*) indicate significant differences from the values of the 6 h P.I. and Ros (Student's t test, $P < 0.01$). (C) Confocal microscopic images of the mouse embryos immunostained with anti-Ser780-pRB antibody (S780). DNA was stained with propidium iodide. One-cell embryos, early 2-cell, and late 2-cell embryos were collected 12, 19, and 30 h P.I., respectively, and immunostained with anti-S780-pRB antibody. The experiment was conducted three times. Similar results were obtained in each case.

Ser807/811 when compared to controls (data not shown). Likewise, injecting cyclin A2 d-siRNA resulted in a marked reduction in Thr356 phosphorylation (Fig. 7). This effect was specific, because it was not observed when rabbit globin d-siRNA was injected (Fig. 7). Moreover, injection of recombinant cyclin A2-CDK2 protein 1 h after insemination resulted in a substantial increase in Thr356 pRB phosphorylation determined 6 h post-insemination as determined by immunocytochemistry (Fig. 8). This effect was not observed when heat-inactivated protein was injected. In toto, results of these experiments indicate that cyclin A2 mRNA recruited following fertilization leads to activation of CDK2.

Discussion

Once EGA initiates, expression of factors involved in transcription could sustain and augment transcription. Nevertheless, initiation of EGA must depend on molecules that are maternally inherited; sperm-derived factors are not involved because EGA occurs in parthenogentically activated eggs. The identity of such factors, however, has remained elusive. The results described here, using a combination of approaches to inhibit or increase CDK2 activity, provide new insights regarding the molecular basis for EGA initiation following fertilization, namely, recruitment of maternal cyclin A2 mRNA following fertilization leads to activation of CDK2 that in turn participates in EGA. It is unlikely that cyclin E binds to and activates CDK2, as in somatic cells (Sherr, 1996), to initiate EGA, because cyclin E is not detected in the mouse 1-cell embryos by immunoblotting (Naito K., unpublished data).

The mechanistic linkage between CDK2 activation by complexing with newly synthesized cyclin A2 and EGA, however, is not resolved. Nevertheless, there are a number of reports demonstrating that cyclin A2-CDK2 can regulate

transcription. For example, cyclin A2-CDK2 can directly activate a transcription factor. Several reports demonstrate that general transcription factor Sp1 can associate with and be phosphorylated by cyclin A2-CDK2 (Fojas de Borja et al., 2001; Haidweger et al., 2001). Phosphorylation of Sp1 increases its DNA binding affinity (Fojas de Borja et al., 2001; Haidweger et al., 2001) and hence could stimulate transcription. Sp1 binding sites, the GC box and the related GT/CACC box are present in many promoters and other regulatory sequences of tissue-specific and ubiquitous genes (Philipsen and Suske, 1999), suggesting that cyclin A2-CDK2 regulates expression of numerous genes via regulating Sp1 activity. Of note is that the amount of phosphorylated Sp1 increases during the 1-cell stage (Worrad and Schultz, 1997).

pRB is also a substrate for CDKs (Sherr, 1996) and pRB phosphorylation is known to modulate transcription (Harbour and Dean, 2000a,b; Nevins et al., 1997; Swanton, 2004; Vandel et al., 2001). pRB can interact with the transcription activation domain of members of the E2F transcription factor family, which modulate the expression of many genes, and thereby inhibit their activity. In addition, pRB can interact with enzymes involved in chromatin remodeling such as histone deacetylases (HDACs). Such an interaction could also lead to repression of transcription. pRB phosphorylation leads to dissociation of pRB from E2F members or release of HDAC, each of which will relieve repression of that particular promoter. Cyclin A2-CDK2 can phosphorylate pRB and results of experiments reported here strongly implicate cyclin A2-CDK2 phosphorylating pRB in the 1-cell embryo during a time consistent with this kinase being involved in EGA, perhaps through the mechanisms described above. Of interest is that an increase in histone acetylation occurs during the time of EGA (Adenot et al., 1997). Current experiments are addressed at determining direct targets of cyclin A2-CDK2 that are involved in initiating EGA.

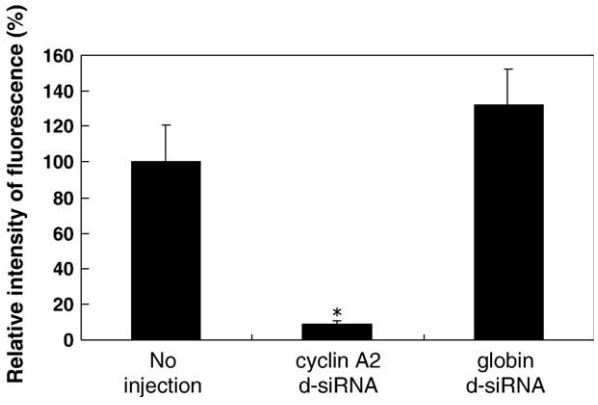

Fig. 7. Effects of cyclin A2 d-siRNA microinjection on the phosphorylation of Thr356 on pRB in the mouse 1-cell embryos. MII stage oocytes were microinjected with mouse cyclin A2 d-siRNA or rabbit alpha-globin d-siRNA. Twelve hours after insemination, they were immunostained with anti-Thr356-pRB antibody. The average value in the control embryos with no injection was set as 100%. The experiments were conducted twice and similar results were obtained in each case. The total numbers of embryos examined were 11, 9, and 10 for no injection, cyclin A2 d-siRNA injection, and globin d-siRNA injection, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the values of the no injection and globin d-siRNA injection (Student's t test, $P < 0.01$).

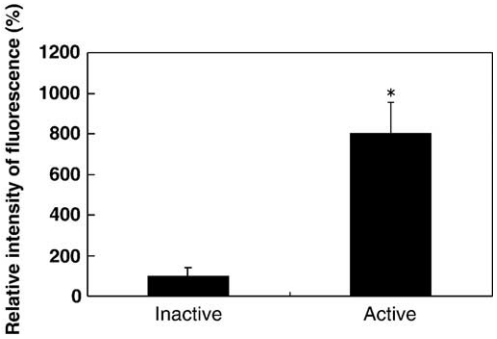

Fig. 8. Effects of cyclin A2-CDK2 protein microinjection on phosphorylation of Thr356 on pRB in the mouse 1-cell embryos. One-cell embryos were microinjected with active or heat-inactivated cyclin A2-CDK2 protein at 1 h after insemination. They were collected 6 h post-insemination and subjected to immunostaining with anti-Thr356-pRB antibody. The average value in the embryos microinjected with inactive cyclin A2-CDK2 protein was set as 100%. The experiments were conducted three times and similar results were obtained in each case. The total numbers of embryos examined were 18 and 24 for inactive protein injection and active protein injection, respectively. The columns and bars represent mean ± SEM. An asterisk (*) indicates significant difference from the value of the inactive cyclin A2-CDK2 injection (Student's t test, $P < 0.01$).

Last, our finding may provide insight into the mechanism for the dramatic reprogramming in gene expression that accompanies EGA with the conversion of a highly differentiated oocyte into totipotent blastomeres. The transcription machinery in the embryo prior to EGA is of maternal origin. So how can EGA lead to dramatic changes in gene expression? By recruiting a maternal mRNA, i.e., cyclin A2, the embryo is able to up-regulate expression of a protein during this transition. And by up-regulating synthesis of cyclin A2, which as described above can modulate the function of proteins involved in expression of a large number of genes, a reprogramming of gene expression may ensue.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.07.012.

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