Factors engaged in reactivation of DNA replication in the nuclei of growing mouse oocytes introduced into the cytoplasm of parthenogenetic one-cell embryos

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ABSTRACT  Mammalian primary oocytes are arrested in the post-replicative G2 phase of the cell cycle. In contrast to other G2 nuclei, the nucleus of the growing mouse oocyte can reinitiate DNA synthesis after transfer by cell fusion under favorable cytoplasmic conditions, created by the parthenogenetic one-cell embryo. In the present study, we used the cell hybrid system to analyze the distribution of proteins involved in DNA re-replication in the oocyte nucleus. We show that this process is preceded by an extensive rearrangement of the insoluble fractions of minichromosome maintenance (MCM) proteins (Mcm2, -6 and –7). We also demonstrate that Cdc6 protein is present in primary growing mouse oocytes freshly isolated from the ovary, in a soluble and insoluble form. In contrast to MCM proteins, the insoluble fraction of Cdc6 was not rearranged in oocyte nuclei reinitiating DNA replication in hybrid cells. The rearrangement of MCM proteins and reinitiation of DNA synthesis occurred in the nuclei, in which the nuclear envelope remained intact. Reinitiation of DNA replication in the oocyte nucleus was sensitive to the inhibition of both CDK activity and polyadenylation of maternal mRNAs, indicating a role of proteins synthesized de novo by the embryo. These results allow us to understand better the mechanisms involved in the reinitiation of DNA replication in growing oocytes.

KEY WORDS: hybrid cell, MCM protein, Cdc6, DNA synthesis, pre-RC

Introduction

For the majority of proliferating eukaryotic cells it is vital to duplicate their DNA only once per cell cycle. For this purpose not only the initiation and termination of DNA synthesis has to be precisely controlled, but also special mechanisms have to be developed to protect the post-replicative G2 cell against undesired, accidental DNA synthesis. The multiprotein pre-replication complexes (pre-RCs), which are build on each replication origin during telophase and the G1 phase (Dimitrova et al., 1999, 2002), and license the chromatin for replication, change their status into the inactive post-replicative complexes while the S phase progresses. This is achieved by gradual detachment from the chromatin of several replication-related proteins, like the Minichromosome Maintenance (MCM) family proteins. They remain in the nucleus, but only in the soluble (chromatin unbound) form (Todorov et al., 1995; Dimitrova et al., 1999; 2002; Nishitani and Lygerou, 2002). Increasing CDK1 (cyclin dependent kinase 1) activity, typical for G2 cells, prevents formation of new pre-RCs during S and G2 phases thus protecting the genome against DNA re-replication. Experimental inhibition of CDK1 activity allows initiation of DNA replication in G2 fibroblasts (Usui et al., 1991). Similar effect was obtained after exposition of the G2 cells to the

Abbreviations used in this paper: h a.a., hours after activation; BSA, bovine serum albumin; Cdc6, cell division cycle-6 homologue; CDK, cyclin dependent kinase; CSK, cytoskeleton buffer; DDK, Dbf4-dependent kinase; 3'-dA, 3'-deoxyadenosine; Dig-11-dUTP, digoxigenin-11-2'-deoxy-uridine-5'-triphosphate; DMSO, dimethylsulfoxide; dU, deoxyuridine; FITC, fluorescein; GV, germinal vesicle; hCG, human chorionic gonadotrophin; Hp1α, heterochromatin protein 1α; MCM, minichromosome maintenance proteins; ORC, origin recognition complex; PBS, phosphate buffered saline; PBSA, Ca2+, Mg2+-free phosphate buffered saline; PFA, paraformaldehyde; PMSG, pregnant mare’s gonadotrophin; PMSF, phenylmethylsulfonyl fluoride; PNu, pronucleus; pre-RC, pre-replication complex; SN, surrounded nucleolus oocyte.

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inhibitor of serine-threonine kinases (Coverley et al., 1996; 1998). Normally the nuclei of somatic and embryonic G2 cells are unable to restart DNA synthesis, even after fusion with S-phase cells (Rao and Johnson, 1970; Czolowska and Borsuk, 2000). New round of chromatin licensing, preceding new round of DNA replication, is possible only after nuclear envelope breakdown and the completion of mitosis with concomitant decrease in the CDK1 activity (reviews: Nurse, 1997; Nishitani and Lygerou, 2002).

Growing oocytes of the mouse are usually considered as cells arrested in the prolonged G2 phase, since the last round of DNA replication occurred in the embryonic life before the entry of female germ cells into meiosis (Lima-de-Faria and Borum, 1962; Peters et al., 1962). Before the next round of DNA synthesis occurs, the primary oocyte must terminate the phase of growth, develop the competence to undergo maturation, pass through the first meiotic division and, after activation by sperm or artificial stimulus, complete the second meiotic division. Only after executing all tasks and transition into the interphase of the first embryonic cell cycle, the new round of DNA replication can be initiated. During the entire period of their ovarian life primary oocytes had to be maintained in a state of repression of DNA synthesis. Like any G2 cells these cells are expected to sustain their chromatin in the unlicensed state, characterized by the absence of chromatin-bound form of proteins engaged in the formation of pre-RCs. Primary growing mouse oocytes appeared, however, unique in this respect. We have demonstrated recently (Siewiach et al., 2007), that some members of MCM family proteins (Mcm2 and Mcm7) are accumulated in growing oocyte nuclei in the insoluble form, being tightly associated with chromatin. This result was surprising, as in the nuclei of somatic cells (Dimitrova et al., 1999) and pronuclei of one-cell mouse embryos (Siewich et al., 2007) the insoluble form of these proteins was observed only in G1 and S phase of the cell cycle. It needs also to be stressed, that the organization of insoluble form of both Mcm2 and Mcm7 in growing oocytes differed from that observed in the female pronuclei of parthenogenetic one-cell embryos. The abundance of these replication-related proteins may partly explain why G2 nuclei of growing oocytes are able to reinitiate DNA replication stimulated by exogenous stimulus. We showed before (Czolowska and Borsuk, 2000) that such stimulus of unknown nature is provided by parthenogenetic one-cell embryos at different phases of the first cell cycle (telophase II/G1, G1/S and S). What is important, in the same experimental conditions (fusion with one-cell embryo) neither the nuclei of fully-grown mouse oocytes (Borsuk, unpublished data) nor the G2 nuclei of embryonic cells (blastomeres of 2-cell embryos; Czolowska and Borsuk, 2000) were capable of reinitiating DNA synthesis. In the present studies we searched for the factors which may be involved in the renewal of DNA replication by G2 nuclei of growing oocytes in the hybrid cells.

As mentioned above, the proper organization of pre-RCs is required for the initiation of DNA replication. For this purpose certain replication-related proteins have to bind sequentially to the chromatin. Beside the MCMs, the Cdc6 (cell division cycle-6 homologue) is the crucial protein of pre-RC. Its binding to the origin recognition complex (ORC) ensures the binding of MCMs. It was documented that Cdc6 protein is absent in fully-grown preovulatory oocytes of the mouse (Lemaître et al., 2004; Anger et al., 2005), but no data concerning the presence or absence of this protein in growing oocytes are available. If present in oocytes in a phase of growth, this protein could also participate in the process of oocyte chromatin licensing.

In the somatic cell cycle the firing of replication origins is impossible without the activation of pre-RCs by two kinases: DDK (Dbf4-dependent kinase) and CDK 2 (review: Nishitani and Lygerou, 2002; Forsburg, 2004). Only in activated pre-RC the MCM complex has the ability to bind Cdc45, another initiator protein. The complex MCM-Cdc45 expresses the activity of DNA helicase and unwinds DNA duplex, what makes the origins of replication accessible for DNA polymerase (Masuda et al., 2003). The role of CDK2 in the activation of pre-RCs in embryonic cells has been at least partly confirmed by Fuchimoto et al. (2001) who showed that inhibition of CDK2 activity in zygotes resulted in significant decrease of the frequency of replicating pronuclei. CDK2 acts together with cyclin A, the regulatory subunit of CDK kinase. Two types of cyclin A, A1 and A2, have been demonstrated in mouse germ cells. Cyclin A1 is typical for meiotically dividing cells and dominates in GV stage and metaphase II oocytes (Sweeney et al., 1996). Cyclin A2 has been detected in fully-grown and in metaphase II oocytes, where it concentrates at the polar regions of the meiotic spindle (Winston et al., 2000, 2001). The level of cyclin A2 increases rapidly after fertilization (Fuchimoto et al., 2001; Winston et al., 2001), due to polyadenylation of maternal cyclin A2 mRNA and its translation (Fuchimoto et al., 2001; Hara et al., 2005). Although the role of this de novo synthesized cyclin A2 in DNA replication in zygotes is controversial, cyclin A2 in a complex with CDK2 can be potential trigger of DNA replication in oocyte nucleus.

In the present studies we analyzed the behavior of replication-related proteins in the growing oocyte nuclei, introduced into one-cell parthenogenetic embryos, and tried to identify the possible factors triggering DNA synthesis in these nuclei. We have found that, on the contrary to fully-grown oocytes (Lemaître et al., 2004; Anger et al., 2005), the Cdc6 protein is present in the nucleus of growing mouse oocyte, freshly isolated from the ovary, in the insoluble (chromatin bound) form. We demonstrated that in hybrid cells the reactivation of DNA synthesis in growing oocyte nuclei occurred without its nuclear envelope breakdown. Reactivation was sensitive to the inhibition of both CDKs activity and polyadenylation of maternal mRNAs, and was preceded by an extensive rearrangement of the insoluble form of MCM proteins but not of insoluble form of Cdc6 protein.

Fig. 1. Schematic representation of the experimental protocol, demonstrating the localization of the fusion procedure (hours after activation, h a.a.) and timing of hybrid cells fixation (hours after fusion) in relation to the phases of the first cell cycle.
Results

Timing of DNA replication in parthenogenetic one-cell embryos and in hybrid cells

Control parthenogenetic one-cell embryos cultured in vitro entered the S phase about 5-6 h after activation (a.a.) and completed replication at 13 h a.a. Fusion of parthenogenetic one-cell embryo with growing oocyte performed 4-7 h a.a. (corresponding to G1 and early S phases of the first embryonic cell cycle, Fig. 1), did not delay significantly the initiation and progression of the S phase by the female pronucleus in hybrid cell. In contrast, activation of DNA synthesis in the nucleus of the growing oocyte in hybrid cell could occur only after initiation of replication by the female pronucleus and usually was observed 2-3 hours later than in the pronucleus. The firing of replication domains proceeded along different pattern than in the pronucleus (Czolowska and Borsuk, 2000; this work). Usually, the first signs of the incorporation of microinjected dU analogue (Dig-11-dUTP) were observed in the regions of heterochromatin surrounding the nucleolus. Spreading of the signal over the chromatin in oocyte (Fig. 2. Left) Distribution of insoluble form of Mcm2, Mcm6 and Mcm7 proteins in hybrid cells fixed 30 – 60 min after cell fusion. (A-D) chromatin stained with propidium iodide (colored in red); (A’-D’) insoluble form of Mcm2, Mcm6 and Mcm 7 proteins (colored in green); (A”-D”) merge of the chromatin and protein pictures. PNu, pronucleus, GV, oocyte nucleus. Bar 20 μm. (A’,B’) In the oocyte nucleus Mcm2 protein forms randomly distributed granules while in the pronucleus it is distributed more evenly on the chromatin and accumulated in the vicinity of the nucleolus-like body (B’, arrow). Numerous Mcm2-positive deposits (A’, arrows) visible in the hemisphere of the hybrid originating from the one-cell embryo; (C’) the insoluble form of Mcm6 is not detected in the oocyte nucleus, while in the pronucleus it is distributed evenly on the chromatin and its signal is strong; (D’) the insoluble form of Mcm7 in the oocyte nucleus appears in the form of irregular patches localized in the regions of heterochromatin (arrows). In the pronucleus Mcm7 is distributed evenly on the chromatin and its signal is very strong.

Fig. 3. (Right) DNA replication and distribution of insoluble form of Mcm2 in hybrids fixed 2 h after cell fusion. (A-D) incorporation of Dig-11-dUTP (DNA replication; colored in red), (A’–D’) distribution of insoluble form of Mcm2 protein (colored in green), (A”–D”) merge of DNA replication and Mcm2. PNu, pronucleus; GV, oocyte nucleus. Bar 20 μm. In the majority of hybrids there was no DNA synthesis in the pronuclei or the synthesis was just initiated (weak signal surrounding the nucleolus-like body) (A). In some hybrids DNA replication in the pronucleus was already in an advanced stage (B,C). Sporadically DNA synthesis was observed also in the oocyte nuclei (D). The insoluble Mcm2 protein was distributed rather uniformly in the pronuclei regardless of the advancement of replication (PNu; A–C’). In the oocyte nuclei the localization pattern of insoluble form of Mcm2 was gradually remodeled. At the beginning it formed granules with blurred outlines (GV; A’). These granules underwent gradual dispersion, and finally few Mcm2-positive species were visible in the nucleus (GV; B’). Finally the insoluble Mcm2 achieved uniform distribution (similar to observed in the pronuclei) with fewer more intensely stained species (compare, belonging to the same hybrid cell, PNu (C’) and GV (D’)).
nucleus occurred at a slow pace, contrary to the pronucleus where the initiation of DNA replication occurred simultaneously in numerous domains and spread rapidly all over the chromatin (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997; this work). To make further description more clear, the age of hybrids will be expressed in hours after fusion (that is the time elapsing from the moment of cell fusion to the moment of hybrid fixation).

The localization pattern of insoluble form of Mcm proteins is remodeled in the nuclei of growing oocytes initiating DNA synthesis in hybrid cells

The localization of insoluble form of Mcm2, Mcm6 and Mcm7 proteins was studied in hybrids between parthenogenetic one-cell embryos and growing oocytes, submitted to cell extraction procedure, developed by Sun et al. (2000) for somatic cells. This treatment removes from the nuclei all unbound proteins, leaving only those, which are attached to the chromatin and/or nuclear matrix. Between 30 and 60 min after the first signs of fusion, many hybrid cells were still deformed and the cytoplasmic compartments of both partners were distinguishable. In these earliest hybrids the female pronuclei were at the G1/S phase of the cell cycle (Fig. 1). The distribution of insoluble form of Mcm2, Mcm6 and Mcm7 proteins in the female pronuclei and oocyte nuclei was similar to observed in control parthenogenetic one-cell embryos and in growing oocytes freshly isolated from the ovaries (Swiech et al., 2007). In the pronuclei the insoluble form of Mcm2, -6 and –7 were distributed evenly on the chromatin. The fluorescent signal of the proteins was strong and usually uniform (Fig. 2 A',B',C',D'). In the oocyte nuclei residing in the majority of these nascent hybrid cells the insoluble form of Mcm6 protein was not detected (Fig. 2C') and the insoluble Mcm7 was present in a form of few, very faintly stained, irregular patches (Fig. 2D', arrows) localized in the regions of heterochromatin. Interestingly, multiple dots of insoluble Mcm2 protein were found in the cytoplasm of this hemisphere of a hybrid, which originated from the parthenogenetic one-cell embryo (Fig. 2A', arrows).

To correlate the behavior of insoluble Mcm2 protein with reactivation of DNA replication in the oocyte nuclei, 23 hybrids were injected with Dig-11-dUTP within 30 min. after fusion and submitted to cell extraction 2 h post fusion. These hybrids were double immunostained for DNA replication and for the presence of insoluble Mcm2 protein. In only 8 hybrids the female pronucleus was replicating and initiation of replication in the oocyte nucleus was detected in the minority of them (2/23 hybrids; Table 1). In all analyzed hybrids, the pattern of localization of insoluble Mcm2 protein in the oocyte nucleus, was remodeled. Three types of reactions were observed: 1. the intensity of fluorescence of Mcm2 globules decreased and their outlines were blurred (Fig. 3A'); 2.

| TABLE 1 |
| THE CORRELATION BETWEEN DNA REPLICATION AND THE PATTERN OF DISTRIBUTION OF INSOLUBLE FORM OF MCM2 PROTEIN IN THE PRONUCLEI AND OOCYTE NUCLEI OF HYBRID CELLs FIXED 2 h AFTER FUSION |
| Replicating nuclei (number of hybrids) | PNu | GV |
| Pattern of distribution of insoluble form of Mcm2 | + | - | + | - |
| globules with blurred outlines | 0 | 15 | 2 | 1 |
| not present or present in few species of low fluorescent signal | 0 | 0 | 9 |
| uniform | 0 | 0 | 11 |
the insoluble form of Mcm2 was either not detected in the oocyte nucleus or it was concentrated in few (2-3), very faintly stained specles (Fig. 3B); 3. strong, uniform staining of the insoluble form of Mcm2, similar to observed in the G1- or S-phase pronucleus, was detected in the oocyte nucleus, accompanied by few (2-3), more intensively stained specles of the protein (Fig. 3D'). This last type of Mcm2 distribution was observed in two hybrid cells, in which the oocyte nucleus was replicating (Fig. 3D). The frequency of hybrids in which these different patterns of Mcm2 occurred in the relation to DNA replication is shown in Table 1. Hybrids in which Mcm2 formed blurred granules occurred with similar frequency as hybrids, in which the signal of the protein was not detected, except of few, faintly stained specles. Hybrid cells, in which oocyte nuclei exhibited more or less uniform labeling of Mcm2, occurred less frequently.

Hybrid cells of the same age as described above (fixed 2 h after fusion), but not injected with Dig-11-dUTP, were submitted to cell extraction procedure and immunostained for the insoluble form of Mcm6 and Mcm7. The insoluble form of both proteins were present in the female pronuclei but were not detected in the oocyte nuclei (Fig. 4), although in earliest hybrids the insoluble form of Mcm7 was observed in a form of discrete patches co-localized with the regions of heterochromatin (compare Fig. 2D' and 4B').

To trace further changes associated with remodeling of insoluble Mcm proteins in oocyte nuclei, hybrid cells were cultured in vitro for 3-6 h (9-11 h a.a.; advanced S phase of the one-cell embryo) before being submitted to the cell extraction. Analysis of a group of 23 hybrids stained for the insoluble Mcm7, and 14 hybrids stained for the insoluble Mcm6 protein revealed their presence in both oocyte nucleus and female pronucleus (Fig. 5).

Fig. 6. DNA replication and distribution of insoluble form of Mcm2 and Mcm6 in hybrids fixed 9-11 h after cell fusion. (A,B) incorporation of Dig-11-dUTP (DNA replication; colored in red), (A',B') distribution of insoluble form of Mcm2 and Mcm6 proteins (colored in green), PNu, pronucleus; GV, oocyte nucleus. (A,B) In hybrids fixed 9-11 h after cell fusion the intensity of the signal of DNA replication in the pronuclei suggested advanced stage of the process. The insoluble Mcm2 protein in the oocyte nucleus was distributed uniformly on the chromatin and the signal of the protein was rather strong (A'). The insoluble Mcm6 protein in oocyte nuclei was accumulated in granules (B'). Numerous Mcm6-positive deposits were observed in the cytoplasm of hybrid cells (arrowheads). The insoluble form of both proteins was present and distributed uniformly in the pronuclei (PNu; A',B').

The Cdc6 protein is present in the nuclei of growing mouse oocytes and its localization pattern is not remodeled in oocyte nuclei initiating DNA replication in hybrid cells

We have next analyzed the distribution of the other compound of pre-RCs, the Cdc6 protein, in the nuclei of growing oocytes freshly isolated from the ovaries, in the pronuclei of one-cell parthenogenetic embryos and in hybrid cells. On the contrary to
the fully-grown oocytes, in which the Cdc6 was never detected (Lemaître et al., 2004; Anger et al., 2005), in growing mouse oocytes this protein was present in both the soluble and the insoluble form. The insoluble protein gave uniform signal over the chromatin, but was also accumulated in the nuclear foci (Fig. 7A', arrows) corresponding to the regions of heterochromatin. In the female pronucleus of one-cell parthenogenetic embryo also both form of Cdc6 protein were detected. The insoluble form was distributed rather homogenously except the regions of higher fluorescence, corresponding to more condensed chromatin (Fig. 7B'). In hybrid cells (fixed 2 h and 6 h after fusion) the distribution of insoluble Cdc6 in oocyte nuclei and female pronuclei remained as in non-fused control oocytes and embryos (Fig. 7C').

The specificity of the anti-Cdc6 antibody was verified by immunostaining of mouse NIH3T3 somatic cells grown in culture (see Supplementary material).

**Activation of DNA synthesis in the oocyte nucleus is not preceded by the nuclear envelope breakdown**

Nuclear envelope breakdown in mitosis is a prerequisite for licensing the chromatin for DNA replication in the subsequent cell cycle. Activation of replication in oocyte nuclei observed in hybrid cells might have been thus a consequence of transitory destabilization of the nuclear envelope in the oocyte, evoked by subjection of the oocytes and one-cell embryos to the electric pulses triggering cell fusion. To exclude this possibility we examined the integrity of the nuclear envelope of oocyte nuclei in hybrid cells by intra-cytoplasmic microinjection of fluorescent dextran (MW 70,000). Penetration of dextran into the nucleus would evidence that the nuclear envelope had been temporarily damaged. In this experiment one-cell embryos at G1 were fused with growing oocytes carrying the labeled dextran. In none of 11 hybrids analyzed, penetration of dextran into the nucleus was recorded (Fig. 8).

These results ascertain us that in our reconstructed hybrid cells reactivation of DNA synthesis in oocyte nuclei is not related to the nuclear envelope breakdown.

**Inhibition of Cyclin Dependent Kinases (CDKs) by roscovitine affects initiation of DNA synthesis in the nuclei of growing oocytes in hybrid cells**

The firing of replication origins requires activation of pre-RC complexes by DDK and CDK kinases. Roscovitine, a potent inhibitor of CDKs was used to verify the potential role of these kinases in the initiation of DNA replication in the nuclei of growing oocytes. Parthenogenetically activated oocytes were transferred to the medium supplemented with roscovitine immediately after activation. The treatment of recently activated one-cell embryos with roscovitine often resulted in the inhibition of the second polar body extrusion and formation of two pronuclei. Only one-cell embryos with well visible pronuclei were used for hybrids formation. Twenty five hybrids, obtained by fusion of growing oocytes with roscovitine-treated one-cell embryos, were injected with dU analogue soon after fusion and cultured in the presence of the inhibitor until 18 h post fusion. In none of these hybrid cells the oocyte nuclei initiated DNA replication (Fig. 9 A,B), while in the majority (in 9 out of 11) of control hybrids, not treated with the CDKs inhibitor, the oocyte nuclei were replicating. Despite the presence of roscovitine, in the majority of hybrid cells (16 out of 25) the female pronucleus (or pronuclei, Fig. 9A) synthesized DNA. The intensive signal of Dig-11-dUTP incorporation was also observed in the pronuclei of all control hybrid cells (non treated with roscovitine).

This experiment demonstrates that, the inhibition of CDK kinases by roscovitine blocks completely the reinitiation of DNA synthesis in oocyte nuclei of hybrid cells. Its effect on the DNA replication in pronuclei was rather moderate.

**Inhibition of maternal mRNA polyadenylation affects the DNA replication in the nuclei of hybrid cells**

Cytoplasmic poly(A) elongation is a key mechanism controlling maternal mRNA translation in early development. Several mRNAs are polyadenylated post-fertilization, (Hwang et al., 1997; Temeles and Schultz, 1997; Oh et al., 2000; Fuchimoto et al., 2001; Sakurai et al., 2005), what may lead to increased translation of proteins, which can be used by the embryo in the first and next cell cycles. The biological significance of most of these mRNAs is unclear. It was demonstrated by Fuchimoto et al. (2001) that mRNA of cyclin A2, a protein directly involved in the regulation of DNA replication is also polyadenylated at the beginning of the first cell cycle. We wanted to verify whether in hybrid cells the cyclin A2 synthesized on newly polyadenylated mRNA plays a role in the initiation of DNA synthesis in the oocyte nucleus. For this purpose one-cell embryos were transferred to the medium supplemented with 3'-deoxyadenosine (3'-dA, conc. 3 mM), the inhibitor of mRNA polyadenylation, soon after activation. These embryos were next used for the formation of hybrid cells with growing oocytes. Resulting hybrids were injected with Dig-11-dUTP and

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**Fig. 8. Examination of the integrity of nuclear envelopes in hybrid cell.** Chromatin stained with chromomycin A (colored in red), dextran (MW 70 kDa) colored in green. PNu, pronucleus; GV, oocyte nucleus. (A-A") two growing oocytes microinjected into the cytoplasm with dextran solution and non-injected one-cell embryo. The differences in the intensity of dextran signal in the cytoplasm of oocytes depended on the amount of injected dextran solution. In one oocyte the nucleus is on the other focal plane. One-cell embryo, which was not injected has no green signal in the cytoplasm. Its chromatin is on the other focal plane. (B-B") distribution of dextran in the hybrid cell. After fusion dextran solution was diffused in the whole hybrid cytoplasm. The lack of dextran in the pronucleus and oocyte nuclei in hybrid cell (B") confirms that the electric pulses did not destabilized their nuclear envelopes.
cultured in the presence of 3′-dA until 18 h post fusion. Control hybrids were cultured in 3′-dA free medium and fixed together with experimental group. In 12 control hybrids analyzed, the intensive signal of Dig-dUTP incorporation was observed in the pronuclei, and in 10 hybrids (83.3%) also in the oocyte nucleus. In contrast, in the majority (11 out of 13) of 3′-dA treated hybrids neither the oocyte nuclei nor the pronuclei were replicating (Fig. 9D). In two hybrids the pronucleus exhibited the signal of Dig-11-dUTP incorporation (Fig. 9C), and in one of them very subtle signal of initiation of DNA synthesis was also present in the oocyte nucleus (not shown).

This results confirm that some proteins (most probably cyclin A2), which in the one-cell embryo are translated on the basis of newly polyadenylated mRNA play an important role in the regulation of DNA replication in the oocyte nuclei introduced experimentally into one-cell embryos.

**Discussion**

Accumulation of principal replication licensing factors (the proteins of ORC and MCM complexes) was demonstrated in fully-grown and maturing amphibian (Lemaitre et al., 2002; 2004; Whitmire et al., 2002) and human (Eward et al., 2004) oocytes. In these studies particular protein fractions (soluble vs. insoluble proteins) were not analyzed. In our recent paper we have shown, that some MCM proteins can be immunodetected already in the nuclei of mouse primordial oocytes, and that they accumulate gradually during the period of oocyte growth (Swiech et al., 2007). Two of these proteins, Mcm2 and Mcm7, were detected in the insoluble and soluble form, while Mcm6 was found only in the soluble form. In the present study we used previously developed experimental system permitting to reactivate replication in growing mouse oocytes (Czolowska and Borsuk, 2000). In this system parthenogenetic one-cell embryo exerts stimulatory influence on DNA replication in growing oocyte. We showed, by the immuno-cytochemical methods, that in hybrid cells the reactivation of DNA replication in the oocyte nucleus was preceded by significant remodeling of localization pattern of insoluble (chromatin-bound) MCM proteins. This was particularly spectacular in the case of Mcm2. In the nucleus of growing mouse oocyte isolated from the ovary this protein accumulates on chromatin in the form of multiple granules (Swiech et al., 2007). In hybrid cells the original pattern of protein distribution was erased, and new pattern of its distribution, characteristic for the pronucleus of the one-cell embryo, has been established in the oocyte nucleus. Remodeling was far less spectacular for the insoluble Mcm7, which is less abundant in growing oocytes. The discrete patches of this protein were removed and soon the insoluble form of the Mcm7 appeared on chromatin, in an uniform pattern. The pool of Mcm6, which in the nucleus of freshly isolated growing oocyte exists only in the soluble form, adjoined the oocyte chromatin before reactivation of DNA synthesis. The transition of Mcm proteins between soluble and insoluble form, reflects changes in their phosphorylation. The soluble form of the proteins (chromatin-unbound, detergent-sensitive) is hyperphosphorylated, while the insoluble form (chromatin-bound, detergent-resistant) is hypophosphorylated (Kimura et al., 1994; Todorov et al., 1995; Madine et al., 2000). How these subtle changes are generated in the hybrid cell, in which the chromatin of the pronucleus must have been already licensed for replication (pre-RCs with attached hypophosphorylated MCMs being established in II/G1 phase) is intriguing and for the moment difficult to explain.

In contrast to the MCM proteins, the Cdc6 protein, another important component of the pre-replication complex, remained unremodeled in the oocyte nuclei residing in hybrids. Cdc6 protein was found to be the key-regulator conferring DNA replication competence in amphibian and mouse zygotes. It is not detected in fully-grown, GV-intact oocytes and its synthesis was reported to begin after the initiation of oocyte maturation (Lemaitre et al., 2002, 2004; Whitmire et al., 2002; Anger et al., 2005). In ovulated, metaphase II mouse oocytes Cdc6 accumulates on chromosomes and meiotic spindle (Anger et al., 2005) or only on chromosomes (Borsuk, unpublished), ensuring the binding of other pre-RCs proteins to the chromatin of the female pronucleus in one-cell embryo. Until now, no data concerning the presence or absence of Cdc6 in the early phases of oogenesis were available. We showed for the first time the presence of Cdc6 protein in the nuclei of growing oocytes. In oocytes freshly isolated from the ovaries, Cdc6 was present in both soluble and insoluble form, the insoluble protein showing distinct pattern of distribution on the chromatin. It colocalized with the chromatin and was concentrated at the regions of heterochromatin. This pattern of Cdc6 distribution was maintained in the oocyte nuclei also in hybrid
cells, independently of the time of culture. Very often the first signs of initiation of DNA replication in oocyte nucleus in hybrid cells were observed at the regions of heterochromatin (Czolowska and Borsuk, 2000, this paper). Recently it was shown that Hp1α protein is present in heterochromatin regions in growing mouse oocytes (Meglicki et al., 2008). The significant co-localization between Hp1α and replication proteins, ORC1, ORC2 and Cdc6 was described in mammalian somatic cells (Auth et al., 2006). Apart from several functions suggested (heterochromatin organization, regulation of transcription, involvement in cytokinesis) Cdc6 and HP1α may mark important replication origins on the oocyte chromatin. Particular organization of the chromatin, together with the presence of S-phase driving molecules (MCMs, Cdc6) may render the nucleus of growing oocyte to activate DNA synthesis under stimulating environment. In fully-grown oocytes, just before initiation of maturation (non transcribing SN type oocytes; Debye et al., 1993; Zuccotti et al., 1995; Bouniol-Baly et al., 1999) the heterochromatin regions in the germinal vesicle (GV) are no more visible, HP1α protein dissociates from the chromatin (Meglicki et al., 2008) and Cdc6 protein is no more detected (Lemaître et al., 2004; Whitmire et al., 2002; Anger et al., 2005; Borsuk, unpublished). Correspondingly, reinitiation of DNA synthesis in germinal vesicle after fusion of fully-grown SN oocyte with one-cell parthenogenetic embryo has never been observed.

To accomplish their role in the initiation of DNA replication, the pre-replication complexes need to be activated. In the normal cell cycle replication is triggered in the late G1 by two activators: DDK kinase, acting locally and CDK kinase, acting globally (review, Nishitani and Lygerou, 2002). CDK2 in the active complex with cyclin E controls the transition between G1 and S phase, while in the complex with cyclin A, and particularly with cyclin A2, controls the progression of S phase. The presence of cyclin E in the one-cell embryo remains controversial (Waclaw and Chatot, 2004; Borsuk, unpublished). Correspondingly, reinitiation of DNA synthesis in germinal vesicle after fusion of fully-grown SN oocyte with one-cell parthenogenetic embryo has never been observed.

In somatic cells accumulation of cyclin A2 in the nucleus is possible only after its binding to CDK2 (Jackman et al., 2002). The presence in the nucleus of an active complex, cyclin A2-CDK2, is indispensable for the initiation of DNA replication. CDK2 is present already in mouse oocytes and its level does not change after fertilization though its role in controlling DNA replication in one-cell mouse embryos has been recently negated (Hara et al., 2005). We found that the treatment of hybrids with roscovitine, the inhibitor of CDKs, substantially decreased activation of DNA replication in the pronuclei and blocked this process totally in the oocyte nuclei. These results, together with the effects observed after the treatment with 3’-dA, suggest that the presence of active complexes cyclin A2-CDK2 could be indispensable for the reinitiation of DNA synthesis at least in the oocyte nucleus in hybrid cells.

The observed changes in the distribution of proteins of the pre-RC complex and the renewal of DNA synthesis in the post-replicative oocyte nucleus forced us to reconsider the position of mouse ovarian oocyte in relation to the normal cell cycle. We tend to the opinion that the growing mouse oocyte is maintained in the stage corresponding rather to G1 than G2 cell regarding its replication competence. Many evidences weigh in favor of this opinion. First, activation of DNA replication in oocyte nucleus proceeds without nuclear envelope breakdown, as we proved in this work. The oocyte nucleus reacts thus to S-phase factors provided by one-cell embryo like a G1 nucleus of a somatic cell fused with a somatic cell in S phase (Johnson and Rao, 1970) or like somatic nucleus in G0 transferred into the one-cell mouse embryo (Borsuk and Maleszewski, 2002). Second, growing oocytes accumulate in their nuclei proteins, which are engaged in the initiation of replication, like Mcm2 and Mcm7 (Swiech et al., 2007). Moreover, the insoluble form of Cdc6, which persists in the nuclei of somatic cells during the entire cell cycle (Alexandrov and Hamlin, 2004), is also detected in the nuclei of growing oocytes. The way of accumulation of insoluble MCM and Cdc6 proteins and the lack of insoluble form of other proteins (like Mcm6) prevents formation of functional pre-RC. These insoluble replication-related proteins are eliminated from the nuclei of fully-grown mouse oocytes preparing for maturation (Cdc6 protein: Lemaître et al., 2004; Anger et al., 2005; MCM proteins: Swiech et al., 2007). Only at this stage the mouse ovarian oocyte can be equalized with G2 cell, in which most of the chromatin licensing proteins accumulate in the soluble fraction and the formation of pre-RC complexes is impossible due to increasing activity of CDK1 kinase (Marangos and Carroll, 2004). Fully-grown mouse preovulatory oocytes are incapable to reinitiate DNA synthesis after fusion with parthenogenetic one-cell embryo (Borsuk, unpublished data). Similarly the nuclei of mouse G2 blastomeres fused with parthenogenetic one-cell embryos (Czolowska and Borsuk, 2000) and nuclei of G2 somatic cells fused with S phase cells (Rao and Johnson, 1970) do not re-initiate DNA replication. All these evidences strongly suggest that mouse oocytes, although arrested in meiotic prophase, for the major part of their life in the ovary express features characteristic for G1 cells, attaining the G2 characteristics just before initiation of maturation.

**Materials and Methods**

Ethical approval for this study was obtained from the Local Ethic
Committee No.1 in Warsaw (Poland). Oocytes were obtained from F1(C57Bl/6 x CBA/H) and F1(C57Bl/10 x CBA/H) females and from F1 females of reverse crosses. The chemicals were purchased from Sigma-Aldrich Co. (Germany) unless stated otherwise.

Growing oocytes
Growing oocytes (diameter 50-60 μm) were obtained by puncturing the ovaries of sexually immature females (9-14 days post partum) in M2 medium (Fulton and Whittingham, 1978). The zona pellucidae were removed with 0.5% Pronase in Ringer’s solution. Zona-free growing oocytes were cultured in M2 medium, under paraffin oil, at 37°C, in the atmosphere of 5% CO₂ in the air.

Ovulated oocytes
Ovulated, metaphase II arrested oocytes, were obtained from adult females induced to ovulate with intraperitoneal injection of 10 IU of pregnant mare’s serum gonadotrophin (PMSG, Folligon) and 10 IU of human chorionic gonadotrophin (hCG, Chorulon) (Intervet, International B.V., Boxmeer, Holland) given 46-52h apart. Oocytes were harvested 16-18 h after hCG injection, treated with hyaluronidase (500 μg/ml in Ca²+- Mg²+-free phosphate buffer saline (PBS)) to remove cumulus cells, washed in M2 medium and cultured in the same medium under standard culture conditions.

Parthenogenetic activation
Ovulated oocytes (18-181/2 h after hCG injection) were activated by exposure to 8% ethanol in M2 for 8 min (Cuthbertson et al., 1981; Cuthbertson, 1983). After washing in M2 they were transferred into droplets of this medium under mineral oil and cultured under standard culture conditions (37.5°C, 5% CO₂ in the air).

Formation of hybrid cells
Parthenogenetic one-cell embryos and growing oocytes were preincubated for a few minutes in phytohemagglutinin (300 μg/ml in bovine serum albumin-free M2 medium) in an agar-coated embryological watch glass and agglutinated into pairs. Pairs were fused using the method of Kubiak and Tarkowski (1985). They were washed twice in 0.25M glucose supplemented with 100 μM CaCl₂ x 2H₂O and 100 μM MgSO₄ x 7 H₂O, and transferred to a fusion chamber filled with the same culture conditions (37.5 oC, 5% CO₂ in the air).

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Inhibition of CDK kinases with roscovitine
Oocytes activated parthenogenetically (see above) were washed in M2 medium and immediately after they were divided into two groups. One (control group) was cultured in the M2 medium, the other was cultured in M2 supplemented with the inhibitor of CDK kinases – roscovitine (conc. 100 μM, prepared from the stock solution of 100 mM in DMSO, the final concentration of DMSO in the culture medium 0.1%). Activated oocytes from both groups were used for hybrids formation (see above). Fusions with growing oocytes (cultured in M2) were performed between 5 and 7 h after activation. Obtained hybrids were cultured in M2 (control group) or in M2 supplemented with roscovitine.

Treatment of hybrids with 3′-deoxyadenosine
Immediately after parthenogenetic activation one-cell embryos were transferred into M2 medium (control group) or to the M2 supplemented with inhibitor of RNA polyadenylation, 3′-deoxyadenosine (3′-dA; cordycepin; conc. 3 mM). 3′-dA is metabolized to 3′-dTTP and incorporated into the poly(A) tail, what inhibits further addition of adenosine and terminates elongation of poly(A) tail. Embryos from both groups were used for formation of hybrids with growing oocytes (non-treated with 3′-dA). Fusions were performed between 5 and 7 h after activation. Hybrid cells were cultured in M2 (control group) or in M2 with 3′-dA.

Detection of DNA replication
Hybrids and control one-cell parthenogenetic embryos were microinjected into the cytoplasm with digoxigenin-11-dUTP (Dig-11-dUTP, 1 mM solution – undiluted commercial product, Roche Diagnostics GmbH, Mannheim, Germany), using an Eppendorf microinjector (Eppendorf, Germany). The injected volume was always less than 2 pl. Hybrids were injected mostly between 30 min. and 2 h after fusion. Control one-cell parthenogenetic embryos were injected like hybrids. Hybrids were cultured for different periods of time after injection, but never longer than up to 11 hours after activation, and either subjected to the cell extraction procedure (see below) or fixed for immunocytochemistry. The hybrids treated with inhibitors (roscovitine or 3′-dA) and their controls were cultured in vitro until 22-24 h after activation. Experimental and control hybrids were processed for immunocytochemistry according to the method of Bouniol et al. (1995). They were fixed in 2% paraformaldehyde (PFA) in PBS₅ for 20 min and after washing in PBS₅ they were permeabilized in 0.2% Triton X-100 in PBS₅ and blocked for at least 60 min in 2% bovine serum albumin (BSA) in PBS₅ before immunostaining. The incorporation of Dig-11-dUTP into newly synthesized DNA was detected by mouse monoclonal anti-digoxigenin primary antibody (Roche Diagnostics GmbH, Mannheim, Germany) followed by FITC-conjugated goat anti-mouse IgH (H+L) secondary antibody (Caltag Laboratories, Burlingame, CA, USA or Invitrogen, Eugene, Oregon, USA).

Cell extraction and detection of insoluble forms of Cdc6 and MCM proteins
To visualize the insoluble form of Mcm2, 6 and 7, and Cdc6 proteins the cells were extracted using the method of Sun et al. (2000). After washing with ice-cold PBS₅ and ice-cold cytoskeleton (CSK) buffer (10 mM Pipes, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂) cells were treated with CSK buffer containing 0.5% Triton X-100, 0.5 mM PMSF, 10 μg/ml of both leupeptin and aprotinin, for 3 min on ice. Immediately after this treatment cells were fixed in 2% PFA in PBS₅ for 20 min and blocked for at least 60 min in 2% BSA in PBS₅ before immunostaining.

The McM2 protein was detected using goat polyclonal anti-Mcm2 antibody (N-19, sc-9839, Santa Cruz Biotechnology Inc., USA) and FITC-conjugated donkey anti-goat IgG (H+L) secondary antibody (Jackson Immunoresearch Laboratories, Inc., Baltimore, USA). The Mcm6 was detected with goat polyclonal anti-Mcm6 (C-20, sc-9843) primary antibody (Santa Cruz Biotechnology Inc., USA) and rabbit anti goat IgG (H+L) secondary antibody conjugated with Alexa Fluor 594 (Invitrogen, Eugene, USA). The Mcm7 protein was detected using mouse monoclonal anti-Mcm7 primary antibody (sc-9966, Santa Cruz Biotechnology Inc., USA) and rabbit anti-mouse IgG (H+L) secondary antibody conjugated with Alexa Fluor 633 (Invitrogen, Eugene, USA). The Cdc6 protein was detected by mouse monoclonal anti-Cdc6 antibody (A21286, Molecular Probes, Eugene, USA) and FITC-conjugated goat anti-mouse IgG (H+L) (Caltag Laboratories, Burlingame, CA, USA) or rabbit anti-mouse IgG (H+L) conjugated with Alexa Fluor 633 (Invitrogen, Eugene, USA) as secondary antibodies.

Microinjection of dextran
Growing oocytes were microinjected into the cytoplasm with the solution of dextran (MW 70 000) conjugated with Rhodamine (Molecular Probes, Eugene, USA), using an Eppendorf microinjector. The solution of dextran was prepared immediately before microinjections from stock solution (10 mg/ml) diluted in 20 mM Pipes buffer (pH 7.4), 0.14 M KCl to give the final concentration of 2 mg/ml. Some of injected oocytes were used for fusion with one-cell embryos, the other served as a control group. Hybrids were cultured for 2.5-3.5 h after fusion in M2 medium, fixed in 2%
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