Transcription Factor E2F and Cyclin E-Cdk2 Complex Cooperate to Induce Chromosomal DNA Replication in Xenopus Oocytes*

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Although no chromosomal DNA replication actually occurs during Xenopus oocyte maturation, the capability develops during the late meiosis I (MI) phase in response to progesterone. This ability, however, is suppressed by Mos proteins and maturation/mitosis promoting factor during the second meiosis phase (meiosis II; MII) until fertilization. Inhibition of RNA synthesis by actinomycin D during early MI prevented induction of the replication ability, but did not interfere with initiation of the meiotic cell cycle progression characterized by oscillation of the maturation/mitosis promoting factor activity and germinal vesicle breakdown. Microinjection of recombinant proteins such as dominant-negative E2F or universal Cdk inhibitors, p21 and p27, but not wild type human E2F-1 or Cdk4-specific inhibitor, p19, into maturing oocytes during MI abolished induction of the DNA replication ability. Co-injection of human E2F-1 and cyclin E proteins into immature oocytes allowed them to initiate DNA replication even in the absence of progesterone treatment. Injection of cyclin E alone, which was sufficient to activate endogenous Cdk2 kinase, failed to induce DNA replication. Moreover, the activation of Cdk2 was not affected under the conditions where DNA replication was blocked by actinomycin D. Thus, like somatic cells, both activities of E2F and cyclin E-Cdk2 complex are required for induction of the DNA replication ability in maturing Xenopus oocytes, and enhancement of both activities enables oocytes to override DNA-replication inhibitory mechanisms that specifically lie in maturing oocytes.

In most animal species, fully grown oocytes undergo maturation in response to hormones, resulting in matured eggs, in which DNA replication is initiated and the early embryonic cell cycle proceeds upon fertilization. During maturation, meiosis takes place to maintain the diploid number of chromosomes which DNA replication is initiated and the early embryonic cell cycle proceeds upon fertilization. During maturation, meiosis is governed by a variety of regulators of G1 phase progression (1–3). Meiotic division is governed by the periodic activation of maturation/mitosis promoting factor (MPF) (3), a complex of Cdc2 kinase and cyclin B (4). In Xenopus, fully grown oocytes are naturally arrested at the first meiotic prophase and progesterone (PG) reinitiates maturation (1). PG stimulation of oocytes induces synthesis of Mos proteins and activates MPF (5), which causes germinal vesicle breakdown ( GVBD ) and progression through the first M phase of the meiotic cell cycle (meiosis I (MI)) (1, 3). MPF activity falls after GVBD, and rises again to initiate the second M phase (meiosis II (MII)) (6, 7). Meiosis is arrested again at MII with high MPF activity. Fertilization down-regulates MPF activity, triggering entry into S phase, and thereby allowing matured eggs to start the early embryonic cell cycle (1, 8). During maturation, protein expression is mostly regulated at the translational level (7, 9–11) by polyadenylation of the pre-existing mRNAs (12), or by unmasking translationally silent mRNAs (13). Inhibition of transcription has little effect on activation of MPF and initiation of meiotic maturation in response to PG (10, 14, 15). Therefore, although relatively high levels of transcription (mostly of rRNA) occur during MI (16, 17), this has been assumed simply to enable enough RNA to accumulate for early embryonic development.

Although no chromosomal DNA replication actually occurs during Xenopus oocyte maturation, the ability to induce DNA replication develops in the late MI phase some time after GVBD in response to PG (18, 19), and is suppressed by Mos proteins and MPF until fertilization (6). Inhibition of MPF re-activation after GVBD by cycloheximide (CHX, a protein synthesis inhibitor), antisense oligonucleotides to Mos or dominant-negative Cdc2 prevents maturing oocytes from progressing into MII, but, in turn, allows them to initiate semi-conservative and aphidicolin (APD, a specific inhibitor of DNA polymerase α)-sensitive DNA replication (6). The factors and molecular mechanisms involved in induction of the DNA replication ability are poorly understood.

In the somatic cell cycle of higher eukaryotes, S phase entry is governed by a variety of regulators of G1 phase progression (20, 21), whose expressions are mainly regulated at the transcriptional level. Two types of G1 cyclin/cyclin-dependent kinase (Cdk) complexes (cyclin D-Cdk4 or Cdk6, and cyclin E-Cdk2) promote G1 progression by phosphorylating key substrates such as the retinoblastoma protein (pRb) (22). The hypophosphorylated form of pRb binds to a family of heterodimeric transcription factors, collectively termed the E2Fs (23), and represses expression of E2F target genes. Phosphorylation of pRb by G1 cyclin-Cdk complexes in late G1 frees these E2Fs, enabling them to transactivate the same genes (24). The E2F-inducible genes include the cyclin E gene (25), thereby creating a positive feedback loop between E2F and cyclin E (cyclin E-Cdk2 complex) allowing a rapid rise of both activities as cells approach the G1/S boundary. In addition, recent findings suggest that E2F and cyclin E-Cdk2 kinase have their own pathways to promote S phase entry (26, 27). On the other hand, the activities of G1 cyclin-Cdk complexes are negatively regulated by two groups of proteins collectively termed Cdk inhibitors (28). The Cip/Kip family of Cdk inhibi-
tors (p21Cip1, p27Kip1, and p57Kip2) bind to cyclin D-Cdk4, cyclin D-Cdk6, cyclin E-Cdk2, and cyclin A-Cdk2 complexes and inhibit their protein kinase activities. INK4 family members (p16INK4a, p15INK4b, p18INK4c, and p19INK4d) specifically target cyclin D-dependent kinases (Cdk4 and Cdk6). Both types of Cdk inhibitors are induced or activated in response to a variety of anti-proliferative stimuli such as DNA damage, contact inhibition, transforming growth factor-β, cell senescence, or terminal differentiation, and arrest cells in G1.

In this paper, we addressed whether any of the G1 regulators of the somatic cell cycle participate in induction of the replication ability in late MI during Xenopus oocyte maturation, focusing particularly on E2F transcription factor. Our findings suggest that, as in the somatic cell cycle, both E2F and cyclin E-Cdk2 activities were required and sufficient to induce DNA replication. However, Cdk2 activation by exogenous cyclin E was not sufficient to initiate DNA replication, suggesting that E2F functions other than activating cyclin E promoter play an important role in S phase induction.

EXPERIMENTAL PROCEDURES

Preparation, Culture, and Microinjection of Xenopus Oocytes—Oocytes were manually dissected and treated with collagenase (2 mg/ml) to remove follicle cells as described (29). Stage VI oocytes were induced to mature by addition of PG (5 μg/ml) GVBD, which usually occurred 2–4 h after PG stimulation, was first judged by the appearance of a white spot and was confirmed by cutting boiled eggs in half with a razor blade. In some cases, oocytes were treated with CHX (100 μg/ml), AMD (100 μg/ml in most experiments unless otherwise noted in the figures), or APD (20 μg/ml) at the times indicated in the figures. Oocytes were microinjected before PG stimulation with 36.8 nl of a solution (20 mM HEPES, pH 7.4, 20 mM NaCl, 4 mM MgCl2, and 1 mM β-mercaptoethanol) containing [α-32P]dCTP (10 μCi/ml, 3000 Ci/mmol; Amersham) and purified recombinant proteins (see below).

Analysis of DNA Synthesis—Basic [α-32P]dCTP-preinjected oocytes were stimulated with PG, treated with CHX 1 h after GVBD, and incubated another 3 h. Individually labeled single oocytes were mixed with 14 non-treated oocytes and homogenized in the presence of protease K (10 μg/ml). After overnight incubation at 37 °C, high molecular weight DNAs were extracted, separated by 1% agarose gel electrophoresis, and analyzed with a Fuji BAS-2000 image analyzer. In some inhibition experiments, the mixture of [α-32P]dCTP and recombinant proteins was injected immediately before PG stimulation. To examine the effect of recombinant proteins on induction of DNA replication, CHX treatment was omitted. To inhibit DNA polymerase α activity, APD was added after injection. For kinetic analyses, oocytes were injected as quickly as possible, and sampled at the indicated times. Relative incorporation of [α-32P]dCTP into chromosomal DNA was determined with a Fuji BAS-2000 image analyzer.

Preparation of Recombinant Proteins—All recombinant proteins, except for p27, were made and purified as glutathione S-transferase (GST) fusion proteins. DN-E2F consists of only the DNA-binding domain of human E2F-1 (30) and other fusion constructs contain the entire coding sequences. As for E2F-1, DP-1, cyclin D1, and p21, each of the cDNAs was cloned into the pGEX vector (Pharmacia). Other GST fusion constructs were as described previously (30–32), or gifts from other investigators. GST fusion proteins were expressed in bacteria with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside for 15 h at 18 °C, purified by glutathione-Sepharose (Pharmacia Biotech Inc.) chromatography, and eluted with 100 mM glutathione. p27 was expressed as a 6x histidine-tagged protein in bacteria (33) under the same inducive conditions as GST fusions, absorbed onto Ni-NTA-agarose (Qiagen) and eluted with 100 mM EDTA and 250 mM imidazole. Eluted samples were extensively dialyzed to remove glutathione, EDTA, or imidazole. In some cases, especially for GST-E2F-1, the recombinant proteins were further purified to homogeneity by gel filtration chromatography. Because truncated GST-E2F-1 proteins contain a DNA-binding domain but not a transactivation domain, it inhibited full-length E2F-1 and was a dominant-negative mutant. As for GST-p21 and GST-DN-E2F, further purification accelerated their degradation and did not improve recovery of the intact proteins. Therefore, we did not go through further purification steps for these two. All recombinant proteins were concentrated by pressure filtration in a buffer containing 20 mM HEPES, pH 7.4, 20 mM NaCl, 4 mM MgCl2, and 1 mM β-mercaptoethanol. Purified recombinant proteins retained their specific activities (e.g. Cdk inhibitory activity, DNA binding activity, etc.) as far as we and others determined, indicating that fusion to GST or 6x histidine tag did not alter the specific functions at least as assessed in vitro. Injections were performed under conditions where the final concentrations of each recombinant protein in Xenopus oocytes did not exceed 0.3 mg/ml using NIH 3T3 fibroblasts. Note that elevated expression of Cdk inhibitors by 3–5-fold was just sufficient to arrest proliferating NIH 3T3 cells in G1. The stability of recombinant proteins in Xenopus oocytes was tested as follows. GST fusion proteins were injected into immature oocytes and were recovered after 4 h by glutathione-Sepharose chromatography. More than 80% of the proteins were recovered, suggesting that injected recombinant proteins were relatively stable in oocytes.

Preparation of Oocyte Extracts, Immunoprecipitation, and In Vitro Kinase Assay—Individual single oocytes treated with various reagents were homogenized on ice in 50 μl of extraction buffer (80 mM β-glycerophosphate, 20 mM EDTA, 15 mM MgCl2, and 1 mM dithiothreitol), centrifuged for 5 min at 4 °C, and the supernatants were stored at −80 °C until further analysis. Extracts were incubated with rabbit antiserum to Xenopus Cdk2 (34) for 1 h on ice, mixed with protein A-Sepharose for 1 h, washed with low and high salt buffers (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1% Triton X-100, and either 100 mM or 1 mM NaCl, respectively), and finally washed with Cdk2 kinase buffer (20 mM HEPES, pH 7.3, 15 mM MgCl2, 5 mM EDTA, and 1 mM dithiothreitol) as described previously. Immunoprecipitated GST proteins were incubated in 25 μl of Cdk2 kinase buffer supplemented with 0.2 mg/ml bovine serum albumin, 0.2 mg/ml histone H1, and 50 μg [γ-32P]ATP (0.12 Ci/mmol) at 25 °C for 20 min. Phosphorylated histone H1 was separated by SDS-polyacrylamide gel electrophoresis and quantified for 32P incorporation by a Fuji BAS-2000 image analyzer. For MPF kinase assay, 5 μl of oocyte extracts (equivalent to 0.1 oocyte) was incubated in a buffer containing 20 mM HEPES, pH 7.5, 10 mM MgCl2, 30 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 0.25 mg/ml histone H1, 62.5 μg [γ-32P]ATP (0.15 Ci/ml) at 25 °C for 15 min. The samples were analyzed as described above.

RESULTS

Inhibition of RNA Synthesis during Early MI Prevents Induction of the DNA Replication Ability—The DNA replication capability is induced in late MI some time after GVBD during Xenopus oocyte maturation, but is masked by Mos proteins and MPF activity through MI until fertilization (6). To visualize the DNA replication ability, we chose to use CHX to block protein synthesis after GVBD. This treatment hampers MPF re-activation and progression to MII (7) and, instead, allows maturing oocytes to incorporate preinjected [α-32P]dCTP into chromosomal DNA (6). DNA synthesis induced by CHX represents replication rather than repair because it is sensitive to APD and occurs only once in a semi-conservative manner (6). To know the exact timing of CHX treatment for the efficient induction of DNA replication, we added CHX before GVBD (at intervals of 1 h after PG stimulation) or after GVBD (at intervals of 15 min) to PG-stimulated oocytes that had been preinjected with [α-32P]dCTP. High molecular weight DNAs were extracted from oocytes 4 h after GVBD and separated in an agarose gel (Fig. 1A). The results clearly indicate that new proteins accumulated until 15 min after GVBD are required for induction of the chromosomal DNA replication ability, whereas synthesis of mitochondrial DNAs was constitutive and was minimally affected by CHX. We decided to treat oocytes with CHX 1 h after GVBD in the following experiments.

Although vigorous transcription (mostly of rRNA) occurs during MI, transcription is thought not to be required for induction of meiotic maturation (14–17). We asked whether RNA synthesis might be involved in induction of the replication ability and examined the effect of AMD on CHX-induced DNA replication in PG-stimulated oocytes. When AMD was added to [α-32P]dCTP-preinjected oocytes at the same time as PG stimulation, no DNA replication was induced by CHX (Fig. 1B, lanes 3–5, for data from individual oocytes). Titration experiments indicated that the minimum concentration required for inhibition of CHX-induced DNA replication was 80 μg/ml
A Little Effect of AMD on Initiation of Meiosis—Effect of AMD on CHX-induced chromosomal DNA replication in Xenopus oocytes. A, immature oocytes preinjected with [α-32P]dCTP were stimulated with PG, and CHX was added at the times indicated on the top of the figure. High molecular weight DNAs were extracted 4 h after GVBD (or 8 h after PG stimulation), separated by 1% agarose gel electrophoresis, and analyzed with a Fuji BAS-2000 image analyzer. B, immature oocytes preinjected with [α-32P]dCTP were stimulated with PG and untreated (−) or treated with CHX (+) 1 h after GVBD. AMD (100 μg/ml for lanes 3–5 and 14–19, 20–100 μg/ml for lanes 8–11 as indicated on the top) was added at the time of PG stimulation (lanes 3–5, 8–11, and 14–16) or CHX treatment (lanes 17–19). Oocytes were harvested 4 h after GVBD and subjected to DNA extraction and analysis. Lanes 3–5, 14–16, and 17–19 show the data from independent oocytes, respectively. The results are representative of two (panel A) or five (panel B) independent experiments using oocytes from as many independent frogs. The positions of mitochondrial (Mt) and chromosomal (Nuclear) DNAs are indicated on the right. (lanes 8–11). To exclude the possibility that AMD might bind to DNA and prevent replication itself, we treated oocytes with AMD (100 μg/ml after GVBD at the same time as CHX and incubated them for an additional 4 h (lanes 17–19). Since CHX treatment initiates DNA replication approximately 2–3 h after GVBD (Ref. 6 and data not shown), there should be enough time for AMD to access chromosomal DNA. The results show that addition of AMD after GVBD had little inhibitory effect on CHX-induced DNA replication, suggesting that AMD did not directly inhibit DNA replication itself, and that early MI-specific transcription is required for induction of the replication ability. In fact, the inhibitory effect was somewhat more distinct when AMD was added in early rather than in mid MI (data not shown). In addition, another inhibitor of transcription, α-amanitin, also prevented induction of the replication ability when added in early MI (data not shown), supporting this conclusion.

Little Effect of AMD on Initiation of Meiosis—One explanation of efficient inhibition of CHX-induced DNA replication by AMD is that transcriptional blockade interferes with initiation or progression of oocyte maturation itself. To test this possibility, we examined GVBD and fluctuation of MPF activities in oocytes stimulated with PG in the presence or absence of AMD. When oocytes were treated with PG, they exhibited a white spot, a mark of GVBD, 2–4 h after stimulation even in the presence of AMD (100 μg/ml) and the kinetics were indistinguishable with or without AMD (Fig. 2A). To confirm that GVBD had certainly occurred, oocytes displaying a white spot were boiled and cut in half with a razor blade. Fig. 2B shows that GV disappeared in oocytes treated with PG and AMD. In a control experiment, oocytes simultaneously treated with PG and CHX at the same time retained the GV intact (7, 10, 11). Oocytes stimulated with PG alone or together with AMD were harvested at intervals of 1 h after GVBD and the MPF activities in their individual extracts were measured. As shown in Fig. 2C, AMD treatment had little effect on activation of MPF and its fluctuation. Therefore, inhibition of transcription during MI did not affect progression of the meiotic cell cycle under the conditions where induction of the DNA replication ability was prevented. Since we did not perform cytological analysis, we were unable to exclude the possibility that transcription during MI is required for chromosome condensation and/or spindle formation.

Universal Cdk Inhibitors and Dominant-Negative E2F Efficiently Inhibit CHX-induced DNA Replication—Transcription factors, E2Fs (E2F/DP heterodimers), are well known to govern the transition from G1 to S phase in the somatic cell cycle of higher eukaryotes (23). We therefore examined whether the DNA replication ability induced during Xenopus oocyte maturation is dependent upon the functions mediated by E2Fs. To suppress all of the E2F activities contained in oocytes, we utilized a dominant-negative E2F (DN-E2F) protein consisting
of only the DNA-binding domain of human E2F-1, which has been previously shown to inhibit S phase entry when introduced into mammalian cells (30). We also used several Cdk inhibitory proteins (28) to test whether any of the G1 cyclin-Cdk (cyclin D-Cdk4 or Cdk6, and cyclin E-Cdk2) activities, which are also well known to govern G1/S transition (22), are involved in this process.

When immature oocytes were injected with recombinant proteins and subsequently treated with PG, DN-E2F very effectively prevented CHX-induced DNA replication (Fig. 3, lane 9), suggesting that certain functions (most probably induction of specific mRNA transcription) mediated by an E2F-like factor contained in Xenopus oocytes is required for induction of the DNA replication ability. Injection of wild-type E2F-1 (E2F-1) had no inhibitory effect (lane 8), precluding the possibility that injection of DNA-binding proteins nonspecifically inhibited DNA replication. The pRb, a negative regulator for some family members of E2F transcription factors (mammalian E2F-1, -2, and -3, but not E2F-4 or -5) (24) did not prevent replication under these conditions (lane 7). We do not yet know whether human pRb is capable of binding to and suppressing Xenopus E2Fs. Or, alternatively, oocytes might express a different set of E2Fs which are free from restraint by pRb. Cloning and characterization of Xenopus E2Fs expressed in oocytes is required to elucidate this matter.

Among Cdk inhibitors, p27Kip1 and p21Cip1, but not p19INK4a, efficiently prevented induction of replication (Fig. 3, lanes 4–6). Since a high dose of p27Kip1 (p21Cip1 as well) is capable of inhibiting MPF activity and thereby preventing GVBD and progression through oocyte maturation, we had performed a series of preliminary experiments and titrated the amount of recombinant Cdk inhibitory proteins. The dosage of Cdk inhibitory proteins used in Fig. 3 was low enough not to show any inhibitory effect on MPF and GVBD. As a matter of fact, oocytes injected with this amount of Cdk inhibitors initiated and progressed through meiotic maturation (data not shown) (34).

As it is well known that p27 Kip1 and p21 Cip1 inhibit Cdk2, Cdk4, and Cdk6 activities and that p19 INK4a is specific for cyclin D-dependent kinases, Cdk4 and -6 (28), these results suggest that CHX-induced replication requires Cdk2 but not
Cdk4 kinase activities. Taken together, these findings suggest that the function (most probably transcription) mediated by an E2F-like factor together with the activity of Cdk2, but not of Cdk4 or -6, is required to induce DNA replication ability during MI.

Cooperation of E2F and Cyclin E-Cdk2 Kinase in Induction of DNA Replication—We injected recombinant E2F components and G1 cyclins separately or in combinations into immature oocytes together with [α-32P]dCTP and activated them with PG to examine their effects on induction of DNA replication without CHX treatments (Fig. 4A).

Wild type E2F-1 or DP-1 alone showed marginally higher efficiencies in inducing 32P incorporation into chromosomal DNA compared with GST control, while co-injection of E2F-1 and DP-1 allowed approximately half of the injected oocytes to initiate DNA replication. Higher dose of E2F-1 alone did not improve the efficiency, suggesting that although expression of the E2F family gene is rate-limiting for activation of the heterodimeric E2F/DP transcription factors in somatic cells, both E2F and DP subunits might be limited in Xenopus oocytes. Alternatively, mammalian E2F components may not bind to Xenopus subunits with high affinity. In addition, a higher dose of E2F-1/DP-1 heterodimers failed to increase the efficiency, indicating that other factors are required to induce DNA replication. In fact, co-injection of p27Kip1 totally abolished DNA replication induced by E2F-1 and DP-1, suggesting that some Cdk (most probably Cdk2) activity is required for DNA replication induced by exogenous E2F/DP heterodimers.

G1 cyclins (cyclin E or cyclin D1) alone exhibited little effect in this assay (see below). However, when E2F-1 and cyclin E were co-injected, more than 90% of oocytes incorporated [α-32P]dCTP into their chromosomal DNA. DNA synthesis induced by E2F-1 and cyclin E was sensitive to APD (data not shown), confirming the initiation of DNA replication rather than DNA repair. DNA replication induced by E2F-1 and cyclin E was sensitive to p27Kip1, suggesting that cyclin E functioned through activation of Cdk. Substitution of cyclin E with cyclin D1 failed to induce replication, again implying the involvement of Cdk2 activity but not that of Cdk4 or Cdk6 in this assay (note that cyclins D2 and D3 but not D1 can activate Cdk2, and that cyclin D1 can activate only Cdk4 and -6 among known Cdks) (32, 36).

Surprisingly, even in the absence of PG stimulation more than 90% of immature oocytes injected with both cyclin E and E2F-1 initiated DNA replication (Fig. 4B), while injection of E2F-1 or cyclin E alone was totally incapable of inducing replication without PG stimulation. Compared with replication induced by PG and CHX, which occurred 7 to 8 h after initial PG stimulation (Ref. 6 and data not shown), DNA replication was induced to occur approximately 5 h after injection of cyclin E and E2F-1 (data not shown). Thus, the activities of an E2F-like factor and cyclin E/Cdk complexes seem to be rate-limiting during MI, and enhancement of both activities accelerated the timing of replication initiation by approximately 2–3 h. These observations indicate that cooperation between selectively activated transcription mediated by E2F and the increase in Cdk activity by cyclin E enabled maturing oocytes to override DNA replication inhibiting machinery such as Mos and MPF, which specifically lies in maturing Xenopus oocytes.

Exogenous Cyclin E Induces Activation of Endogenous Cdk2 but Not DNA Replication—GST-cyclin E fusion proteins were injected into immature oocytes and were recovered with glutathione-Sepharose beads after 2 h incubation in the absence of PG. Fig. 5A shows that its associated kinase activity was evident when assayed in vitro using histone H1 as a substrate, while little activity was observed in a GST control. The extracts from oocytes injected with GST-cyclin E were immunoprecipitated with antisera specifically raised against Xenopus Cdk2 (kindly provided by Drs. Furuno and Sagata) (34) and subjected to in vitro kinase assay using the same substrate. As seen in Fig. 5B, endogenous Xenopus Cdk2 was activated after injection of cyclin E proteins. Approximately 20 times more lysates were used in the experiment showed in Fig. 5, panel A, than in panel B and the calculation of the specific activities suggested that the GST-cyclin E-associated kinase activities were mostly due to Cdk2 kinase. Moreover, in the lysates where the Cdk2 activity was evident, we failed to detect the MPF (Cdc2 kinase) activity (negative data not shown). Thus, exogenous cyclin E proteins selectively activated Xenopus Cdk2, but not Cdc2.

Injection of cyclin E, although it effectively activated endogenous Cdk2 kinase, failed to induce DNA replication (Fig. 4, A and B). But co-injection with E2F-1 proteins efficiently induced replication in the presence or absence of PG stimulation. Co-injection of E2F-1 did not further increase endogenous Cdk2 activity. Therefore, these results indicate that the Cdk2 activation by cyclin E was not sufficient to induce chromosomal DNA replication in this particular system.

Endogenous Cdk2 Activation Does Not Depend Upon Transcription—Since cyclin E-Cdk2 activity was required to induce

**Fig. 5. Exogenous cyclin E activates Cdk2 in Xenopus oocytes.** A, GST-cyclin E (lane 1) or GST (lane 2) proteins injected into 10 immature oocytes were recovered with glutathione-Sepharose beads and subjected to in vitro kinase assay using histone H1 as a substrate. B, extracts (equivalent to 0.5 oocyte) from oocytes untreated (lanes 1 and 2) or injected with cyclin E proteins (lanes 3 and 4) were immunoprecipitated with normal rabbit serum (NRS, lanes 1 and 3) or with rabbit antiserum to Xenopus Cdk2 (Anti-Cdk2, lanes 2 and 4) and the immunoprecipitates were subjected to in vitro histone H1 kinase assay. The results were analyzed with a Fuji BAS-2000 image analyzer. Note that 20 times more lysates were used in panel A than in panel B and that exposure time was 10 times longer in panel B than in panel A.

**Fig. 6. AMD treatment does not affect the kinetics of Cdk2 activation in response to PG.** Oocyte maturation was induced to occur by stimulation of PG in the absence (−AMD) or presence (+AMD) of AMD. Oocytes were harvested at the indicated times and analyzed for Cdk2 kinase activity in vitro using histone H1 as a substrate after immunoprecipitation of each extract (equivalent to 10 oocytes) with specific antiserum to Xenopus Cdk2. GVBD occurred 4 h after PG stimulation. We repeatedly obtained essentially similar results with other independent experiments.
DNA replication and induction of the replication capability dependent upon transcription, we examined whether Cdk2 activation in PG-stimulated oocytes requires transcription. To do this, we treated oocytes with PG in the presence or absence of AMD and sampled them at various times. *Xenopus* Cdk2 proteins were immunoprecipitated from their extracts with Cdk2-specific antiserum and subjected to in vitro kinase assay. Fig. 6 shows that Cdk2 activity began to increase after GVBD, which occurred 4 h after PG stimulation, and gradually increased during MII, and that this activation was not affected by the presence of AMD. In somatic cells, cyclin E is one of the E2F-inducible genes and Cdk2 activation is under the control of E2F-mediated transcription. The fact that Cdk2 was activated in transcription-inhibited *Xenopus* oocytes suggests that expression of cyclin E, the predominant regulatory partner to Cdk2 in maturing oocytes (35), is regulated by an unique mechanism during maturation, possibly by polyadenylation of cyclin E mRNAs. The results also indicate that blockade of DNA replication by AMD was not due to inhibition of Cdk2 activation. Taken together, induction of the DNA replication ability in *Xenopus* oocytes requires Cdk2 activity and the activation of an unknown pathway which presumably involves E2F-like factors.

**DISCUSSION**

Initiation of *Xenopus* oocyte maturation, which is triggered by PG, depends upon protein synthesis and phosphorylation but not upon transcription (7, 9–11, 14, 15). However, vigorous RNA synthesis (mainly rRNA) occurs during MII until some time after GVBD (16, 17, 37). The physiological meaning of this is not clear, and the only explanation is that oocytes need to accumulate RNAs, which will be utilized after fertilization because transcription does not occur during the early embryonic cell cycle (38, 39). In the present study, we showed that transcription during MI presumably mediated by an E2F-like factor contained in *Xenopus* oocytes is responsible for induction of the ability to replicate chromosomal DNA. This ability, although induced during MI, is suppressed during the meiotic cell cycle by Mos proteins and MPF (6), and possibly becomes activated upon fertilization. E2F-mediated transcription alone is not sufficient to induce the replication ability and the activation of Cdk2 is also required. Since Cdk2 associates predominantly with cyclin E in oocytes (35) and we found that induction of Cdk2 activity occurred in the presence of AMD (Fig. 6), Cdk2 activation is probably governed only by the expression of cyclin E in a transcription-independent manner. Thus, the signals triggered by PG may activate three independent pathways: (i) activation of Mos and MPF which leads to initiation of meiotic cell cycle; (ii) activation of cyclin E-Cdk2 kinase complexes; and (iii) induction of specific transcripts probably mediated by *Xenopus* E2F-like factors. The latter two pathways can be uncoupled from the meiotic maturation process, and cooperate with each other to develop the DNA-replication inducing activity during MI, which will probably play an essential role in initiating S phase after fertilization. However, in *Rana pipiens* oocytes, it has been reported that transcriptional inhibition prevents chromosome condensation (15). As we did not perform cytological analysis, it is possible that transcription during early MI is also required for chromosome condensation in *Xenopus* oocytes.

In the somatic cell cycle, E2F activity is induced in late G1 and declines after entry into S phase on phosphorylation (40, 41) or degradation (42, 43) of the subunits. This down-regulation of E2F activity is essential for cells to progress through and exit S phase (44). Therefore, in maturing *Xenopus* oocytes, E2F activity may diminish before the exit from pre-meiotic S phase. In this case, E2F proteins must be induced some time after pre-meiotic S phase and before fertilization, because E2F activity and transactivation of its target genes should be required for entry into S phase after fertilization. Since chromosomal DNA is kept condensed and little transcription is observed during MII (37), early MI seems to be the only period when E2F and its responsive genes are transcribed. Our results shown here are consistent with this idea.

Dobrowolski *et al.* (30) have reported that introduction of the same DN-E2F protein as was used in our study into somatic mammalian cells inhibits S phase entry induced by adenovirus E1A or by wild-type E2F-1, but not by serum stimulation. This result could be due to serum affecting multiple proliferation pathways, only one of which involves E2F-1. However, in this particular experiment, cells were stimulated with serum 16 h after DN-E2F injection, while E1A or wild type E2F-1 was co-injected simultaneously with DN-E2F. Moreover, they suggest that this DN-E2F protein is quite unstable in mammalian cells. Therefore it is reasonable to assume that DN-E2F proteins are already cleared from the injected cells in late G1. In maturing *Xenopus* oocytes, we showed that injected GST-fusion proteins were relatively stable (more than 80% of the proteins were intact after 4 h). Thus, the discrepancy of the result is not due to the type of stimulant used to induce S phase, but mostly due to the experimental system; injection of this DN-E2F molecule is more effective in *Xenopus* oocytes than in mammalian cells.

Requirement of E2F and cyclin E-Cdk2 kinase in induction of DNA replication is commonly observed in a variety of systems (30, 45–47). E2F and cyclin E-Cdk2 complexes can activate each other in somatic mammalian cells (21) because the cyclin E gene is one of the E2F target genes and cyclin E-Cdk2 kinase enhances activation of E2F by phosphorylating pRb. However, evidence has recently accumulated that E2F and cyclin E-Cdk2 complexes have their own separate pathways to promote initiation of S phase (26, 27). Using *Xenopus* oocytes, we found that Cdk2 activation occurred in the presence of AMD and that exogenous cyclin E, which activated endogenous Cdk2, required E2F to initiate DNA replication. Therefore, in this system, an E2F-like factor and cyclin E-Cdk2 complex are independently activated in response to PG, but they cooperate with each other in induction of the DNA replication ability probably by activating separate pathways specific to each of them. Thus, the *Xenopus* oocyte system seems to be the ideal system with which to investigate the downstream pathways specific to either E2F or cyclin E-Cdk2 kinase.

It seems likely that in maturing oocytes certain specific factors and regulatory mechanisms different from those in the somatic cell cycle govern the progression of cell cycle. For example, MPF activation is governed by Mos proteins whose expressions are highly restricted to ovary (5). It is possible that new mechanisms regulating DNA replication exist in oocytes. E2F activities have been reported in eggs and early embryonic cells (48) and E2F molecules mostly seem to exist in free form; they appear to form complexes neither with Rb-family pocket proteins nor with Cdkks. However, it is well known that transcription does not actually occur until the mid-blastula stage of embryos (38, 39). Therefore, it has to be a Rb-free mechanism which negatively regulates E2F-mediated transcription during early embryogenesis. We failed to suppress E2F-mediated function and CHX-induced DNA replication by introduction of recombinant human pRb. Although it is possible that mammalian pRb does not functionally interact with *Xenopus* E2Fs with high affinities, this would imply that E2Fs contained in oocytes are free from restraint by pRb. In fact, some forms of mammalian E2Fs such as E2F-4 and -5 are not under the control of pRb (24). Whatever the mechanisms, it is necessary to identify all of
the E2F molecules expressed in maturing *Xenopus* oocytes and characterize them in terms of regulation of DNA replication. Moreover, since in maturing *Xenopus* oocytes protein expression is mostly regulated by polyadenylation of the pre-existing mRNAs (12), selectively enhanced transcripts induced by exogenous transcription factors should be identified with less technical difficulties than in somatic cell systems. Therefore, this system might be suitable for identifying the E2F-target genes closely coupled with initiation of DNA replication from among a number of candidate genes whose expressions are under the control of E2Fs (23, 49, 50). The proteins encoded by such genes should be the link between G1 regulators and the replication control of E2Fs (23, 49, 50).

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