using a C_{18} column and detection at 254 nm, after pre-column derivitization with phenylisothiocyanate[30].

$^{36}Cl^-$ fluxes in eriC-reconstituted liposomes

We carried out overexpression, purification and reconstitution of eriC as described[4]. Influx of $^{36}Cl^-$ into liposomes comprising *E. coli* polar lipids (Avanti, 25 mg ml^{-1}) and eriC (1.5 μg mg^{-1} lipid) was carried out under concentrative uptake conditions[4], except that solutions were buffered with 25 mM citrate/phosphate and were adjusted to the desired pH with NaOH.

Received 2 May; accepted 27 June 2002; doi:10.1038/nature01000.

1. Milkman, R. An *Escherichia coli* homologue of eukaryotic potassium channel proteins. *Proc. Natl Acad. Sci. USA* **91,** 3510–3514 (1994).
2. Schrempf, H. *et al.* A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*. *EMBO J.* **14,** 5170–5178 (1995).
3. Jiang, Y. *et al.* The open pore conformation of potassium channels. *Nature* **417**, 523–526 (2002).
4. Maduke, M., Pheasant, D. J. & Miller, C. High-level expression, functional reconstitution, and quaternary structure of a prokaryotic ClC-type chloride channel. *J. Gen. Physiol.* **114,** 713–722 (1999).
5. Armstrong, N., Sun, Y., Chen, G. Q. & Gouaux, E. Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature* **395,** 913–917 (1998).
6. Ren, D. *et al.* A prokaryotic voltage-gated sodium channel. *Science* **294,** 2372–2375 (2001).
7. Doyle, D. A. *et al.* The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* **280,** 69–76 (1998).
8. Mindell, J. A. & Maduke, M. ClC chloride channels. *Genome Biol.* **2,** 3000.1–3000.6 (2001).
9. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T. & MacKinnon, R. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* **415,** 287–294 (2002).
10. Mayer, M. L., Olson, R. & Gouaux, E. Mechanisms for ligand binding to GluR0 ion channels: crystal structures of the glutamate and serine complexes and a closed apo state. *J. Mol. Biol.* **311,** 815–836 (2001).
11. Lin, J., Lee, I. S., Frey, J., Slonczewski, J. L. & Foster, J. W. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. *J. Bacteriol.* **177,** 4097–4104 (1995).
12. Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F. & Foster, J. W. Control of acid resistance in *Escherichia coli*. *J. Bacteriol.* **181,** 3525–3535 (1999).
13. Small, P. L. & Waterman, S. R. Acid stress, anaerobiosis and gadCB: lessons from *Lactococcus lactis* and *Escherichia coli*. *Trends Microbiol.* **6,** 214–216 (1998).
14. Merrell, D. S. & Camilli, A. Acid tolerance of gastrointestinal pathogens. *Curr. Opin. Microbiol.* **5,** 51–55 (2002).
15. Foster, J. W. & Moreno, M. Inducible acid tolerance mechanisms in enteric bacteria. *Novartis Found. Symp.* **221,** 55–69 (1999).
16. Hersh, B. M., Farooq, F. T., Barstad, D. N., Blankenhorn, D. L. & Slonczewski, J. L. A glutamate-dependent acid resistance gene in *Escherichia coli*. *J. Bacteriol.* **178,** 3978–3981 (1996).
17. De Biase, D., Tramonti, A., Bossa, F. & Visca, P. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. *Mol. Microbiol.* **32,** 1198–1211 (1999).
18. Purdy, M. D. & Wiener, M. C. Expression, purification, and initial structural characterization of YadQ, a bacterial homolog of mammalian ClC chloride channel proteins. *FEBS Lett.* **466,** 26–28 (2000).
19. Gutknecht, J. & Walter, A. Transport of protons and hydrochloric acid through lipid bilayer membranes. *Biochim. Biophys. Acta* **641,** 183–188 (1981).
20. Rychkov, G. Y., Pusch, M., Roberts, M. L., Jentsch, T. J. & Bretag, A. H. Permeation and block of the skeletal muscle chloride channel, ClC-1, by foreign anions. *J. Gen. Physiol.* **111,** 653–665 (1998).
21. Maduke, M., Miller, C. & Mindell, J. A. A decade of ClC chloride channels: Structure, mechanism, and many unsettled questions. *Annu. Rev. Biophys. Biomol. Struct.* **29,** 411–438 (2000).
22. Piwon, N., Gunther, W., Schwake, M., Bosl, M. R. & Jentsch, T. J. ClC-5 Cl^--channel disruption impairs endocytosis in a mouse model for Dent's disease. *Nature* **408,** 369–373 (2000).
23. Kornak, U. *et al.* Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* **104,** 205–215 (2001).
24. Li, X., Wang, T., Zhao, Z. & Weinman, S. A. The ClC-3 chloride channel promotes acidification of lysosomes in CHO- K1 and Huh-7 cells. *Am. J. Physiol. Cell Physiol.* **282,** C1483–C1491 (2002).
25. Stobrawa, S. M. *et al.* Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* **29,** 185–196 (2001).
26. Lin, J. *et al.* Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.* **62,** 3094–3100 (1996).
27. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA* **97,** 6640–6645 (2000).
28. Miller, J. H. *A Short Course in Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992).
29. Wang, R. F. & Kushner, S. R. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100,** 195–199 (1991).
30. Heinrikson, R. L. & Meredith, S. C. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.* **136,** 65–74 (1984).

Acknowledgements We are grateful to S. Lovett and V. Sutera for help with gene deletions; to C. Williams for technical assistance; and to C. Nimigean for comments on the manuscript.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to C.M. (e-mail: cmiller@brandeis.edu).

Competence to replicate in the unfertilized egg is conferred by Cdc6 during meiotic maturation

Jean-Marc Lemaître, Stéphane Bocquet & Marcel Méchali

Institute of Human Genetics, CNRS, Genome Dynamics and Development, 141 Rue de la Cardonille, 34396 Montpellier Cedex 5, France

Meiotic maturation, the final step of oogenesis, is a crucial stage of development in which an immature oocyte becomes a fertilizable egg[1]. In *Xenopus*, the ability to replicate DNA is acquired during maturation at breakdown of the nuclear envelope[2] by translation of a DNA synthesis inducer that is not present in the oocyte[2,3]. Here we identify Cdc6, which is essential for recruiting the minichromosome maintenance (MCM) helicase to the pre-replication complex, as this inducer of DNA synthesis. We show that maternal *cdc6* mRNA but not protein is stored in the oocyte. Cdc6 protein is synthesized during maturation, but this process can be blocked by degrading the maternal *cdc6* mRNA by oligonucleotide antisense injections or by translation inhibition. Rescue experiments using recombinant Cdc6 protein show that Cdc6 is the only missing replication factor whose translation is necessary and sufficient to confer DNA replication competence to the egg before fertilization. The licence to replicate is given by Cdc6 at the end of meiosis I, but the cytostatic factor (CSF) pathway, which maintains large amounts of active Cdc2/Cyclin B2, prevents the entry into S phase until fertilization.

In most species, oocytes arrest at the prophase stage of the first meiotic division and are not fertilizable. Hormone stimuli induce the resumption of meiosis, with completion of meiosis I and arrest in meiosis II at the metaphase stage in vertebrates. At this stage, the mature oocyte can be fertilized and embryonic development initiated. Analysis of replication initiation factors present in the oocyte showed that Cdc6, an essential factor involved in the assembly of the MCM helicase complex at DNA replication origins[4,5], was undetectable in fully grown oocytes but present in mature oocytes and activated eggs (Fig. 1a). By contrast, other initiation proteins analysed were present in the oocyte (Fig. 1b), either nearly exclusively in the nucleus (MCM subunits, RPA70 and RPA34), or also in the cytoplasm (Geminin, Cdt1, Cdc7, ORC and Cdc45; see Supplementary Information Fig. 1). The electrophoretic migration of Cdc6 shifts downwards in interphasic egg extracts (Fig. 1a), in agreement with previous observations[6–8].

Although Cdc6 was absent in the oocyte, we detected a relatively large amount of the protein, about 5 ng, in each unfertilized egg, which is comparable to the value of 3 ng reported previously[6]. As Cdc6 is an essential factor for replication licensing, we next determined at what point in maturation it was accumulated. In maturation induced by progesterone (Fig. 1c, d), Cdc6 protein became detectable soon after germinal vesicle breakdown (GVBD; 3 h after progesterone addition), when MCM4 is phosphorylated by maturation-promoting factor (MPF) or cdc2/cyclin B[9,10]. Inhibition of protein synthesis by cycloheximide added before GVBD, but not 1 h afterwards, resulted in the absence of Cdc6 in the egg (Fig. 1d). These data show that Cdc6 protein is missing in the oocyte (Fig. 1b) and starts to accumulate during maturation.

Although Cdc6 protein was absent in the oocyte, northern blots showed that *cdc6* mRNA was stored as a maternal mRNA during oogenesis (Fig. 2a). In addition, we detected a shift in electrophoretic mobility of the *cdc6* mRNA soon after GVBD (Fig. 2a), consistent with a polyadenylation process occurring during maturation. This shift was still detected if cycloheximide was added 1 h after GVBD, but was extremely reduced if cycloheximide was added

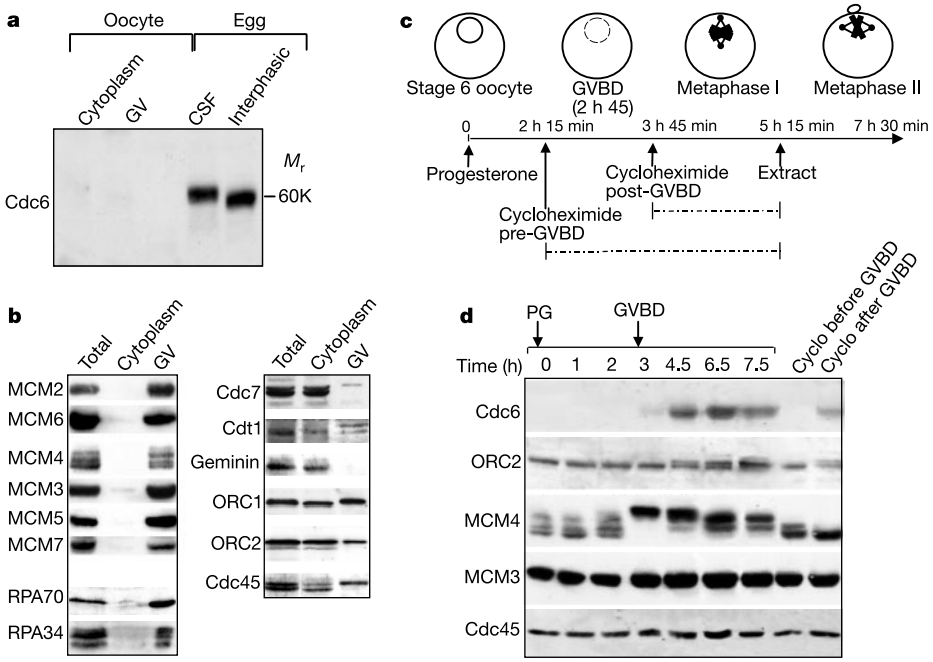

Figure 1 Cdc6 is missing in *Xenopus* oocytes but accumulates during oocyte maturation soon after GVBD. Total oocyte, cytoplasmic and germinal vesicle (GV) fractions, low-speed CSF and interphasic extracts were prepared and analysed by SDS–PAGE. Immunoblots were revealed with antibodies directed against Cdc6 (**a**) and other proteins of the pre-replication complex (**b**). **c**, Stage VI oocytes were induced to mature by adding progesterone (PG). Some oocytes were treated with cycloheximide either 30 min before GVBD or 1 h after GVBD, and collected at the metaphase I/metaphase II transition (2.5 h after GVBD). **d**, Extracts from five oocytes were separated by SDS–PAGE and examined by immunoblotting.

30 min before GVBD, consistent with the respective absence and presence of Cdc6 protein in maturing oocytes (Fig. 1d). Actinomycin D present during maturation did not inhibit this shift (Fig. 2a), suggesting that synthesis of Cdc6 is regulated at the translational level by polyadenylation of a maternal *cdc6* mRNA pool. In addition, the accumulation of Cdc6 protein in the egg was blocked by injecting antisense *cdc6* oligonucleotides into the oocyte before maturation, but not by injecting the corresponding sense oligonucleotides (Fig. 2b). These results suggest that synthesis of Cdc6 protein during oocyte maturation relies on translational control of *cdc6* mRNA stored in the oocyte.

To determine whether Cdc6 was the missing replication factor sufficient to confer the competence for DNA replication to the egg, we examined whether recombinant Cdc6 protein was sufficient to provide this competence. We purified recombinant Cdc6 expressed from baculovirus-infected cells and showed that it was active in rescuing DNA replication in *Xenopus* egg extracts that were depleted of Cdc6 through a purified polyclonal antibody specific to Cdc6 (Methods and Supplementary Information Fig. 2). We used this purified recombinant protein in the *in vivo* rescue experiments described below.

The activity of the missing DNA synthesis inducer that is synthesized during maturation is normally repressed by MPF to prevent DNA replication between meiosis I and meiosis II (ref. 3). Addition of cycloheximide 1 h after GVBD prevented the reaccumulation of MPF and consequently induced unscheduled DNA replication between meiosis I and meiosis II (ref. 3). To address whether this induced ability to replicate required the synthesis of

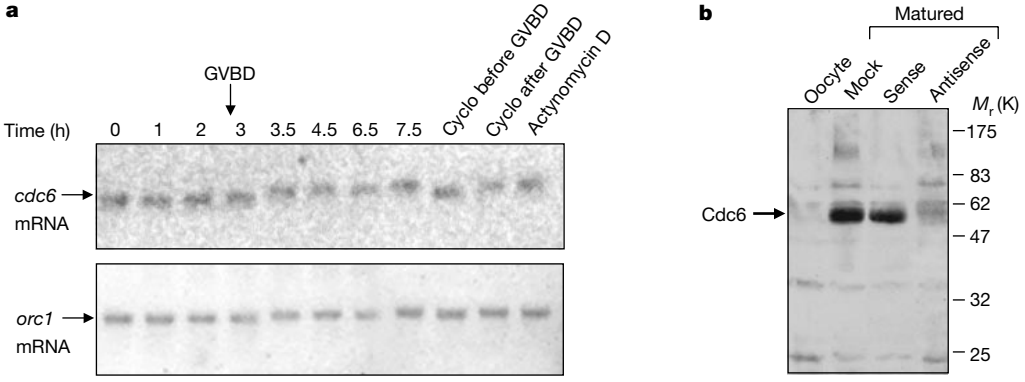

Figure 2 *cdc6* mRNA stored in the oocyte is translated after GVBD. **a**, Oocytes were induced to mature by adding progesterone (PG). Some oocytes were also treated with cycloheximide as described in Fig. 1. RNA was analysed by northern blot. **b**, Stage VI oocytes were injected with 50 ng of a 25-nucleotide sense or the corresponding antisense *cdc6* oligonucleotide, or water as a control (mock), and induced to mature by progesterone. Matured oocytes were collected 12 h after progesterone stimulation and analysed for Cdc6 protein.

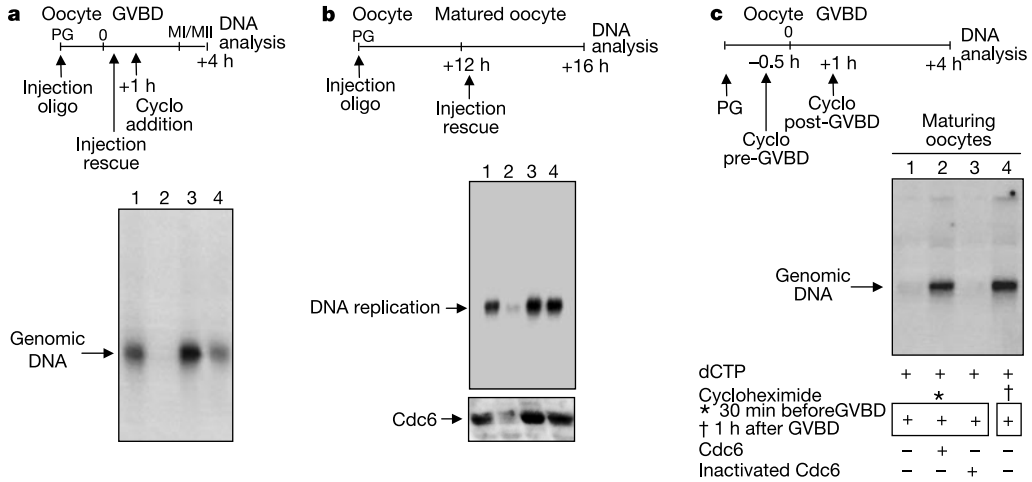

Figure 3 Cdc6 is the DNA replication induced activity acquired *in vivo* during maturation. **a**, Oocytes injected with 25 nl of a 2 mg ml^{-1} solution of purified *cdc6* sense oligonucleotide (lane 1) or the corresponding antisense oligonucleotide (lanes 2 and 3), and control oocytes injected with water (lane 4) were induced to mature. Rescue experiments were assayed by a second injection soon after GVBD that contained 0.25 μCi of [α-^{32}P]dCTP (all lanes) and either boiled inactivated Cdc6 (lane 2) or intact purified Cdc6 (lane 3). Maturing oocytes were incubated in cycloheximide added 1 h after GVBD to test for ability to induce DNA replication between meiosis I and meiosis II. DNA synthesis was analysed 4 h after GVBD as described[3] by agarose gel electrophoresis. **b**, Oocytes injected with water (lane 1), antisense oligonucleotides (lanes 2 and 3) or sense oligonucleotides (lane 4) were allowed to mature fully for 12 h. DNA replication was analysed by injecting 0.25 μCi of [α-^{32}P]dCTP in the absence (lanes 1, 2 and 4) or presence (lane 3) of recombinant Cdc6 protein. DNA synthesis was analysed 4 h after injection by agarose gel electrophoresis. Injected oocytes were also analysed by immunoblot with Cdc6 antibody (bottom). **c**, Oocytes induced to mature were treated with 250 μg ml^{-1} cycloheximide added 30 min before GVBD (*) or 1 h after GVBD (†). Maturing oocytes were injected soon after GVBD with 0.25 μCi of [α-^{32}P]dCTP without (lanes 1 and 4) or with active Cdc6 (lane 2) or inactivated Cdc6 (lane 3). DNA synthesis was analysed 4 h after GVBD by agarose gel electrophoresis.

Cdc6 during maturation, we first determined whether *cdc6* mRNA depletion by antisense oligonucleotide could prevent this unscheduled replication. Addition of cycloheximide 1 h after GVBD induced DNA replication between meiosis I and meiosis II, as previously reported[3], but this induction was inhibited by *cdc6* antisense oligonucleotides (Fig. 3a). In addition, injecting recombinant Cdc6 rescued the induction of unscheduled replication (Fig. 3a).

We next determined whether Cdc6 was sufficient to confer competence to replicate *in vivo* to the matured oocyte. After maturation *in vivo*, parthenogenetic activation triggered by micropipette injection induces DNA replication (refs 2, 11, 12, and Fig. 3b, lane 1). Injecting *cdc6* antisense oligonucleotides into the oocyte before maturation inhibited DNA replication (Fig. 3b, lane 2), but microinjection of Cdc6 protein after maturation was sufficient to rescue DNA replication (Fig. 3b, lane 3) with an efficiency similar to control oocytes treated with sense oligonucleotides (Fig. 3b, lane 4). We conclude that Cdc6 is the missing factor synthesized during oocyte maturation, sufficient to confer both replication-induced ability between meiosis I and meiosis II and competence to replicate in the unfertilized egg.

Previous work has shown that maturing oocytes can replicate DNA after meiosis I by cycloheximide treatment 60 min after GVBD but not 30 min before GVBD[3]. We reproduced these data, confirming that competence for DNA replication *in vivo* is acquired shortly after GVBD and is dependent on translation (Fig. 3c, lanes 1 and 4). We also showed that microinjection of Cdc6 after translation inhibition 30 min before GVBD is sufficient to rescue induced-replication ability between meiosis I and meiosis II (Fig. 3c, lanes 1–3).

These *in vivo* observations could be reproduced *in vitro*, in oocyte extracts prepared between meiosis I and meiosis II (refs 13, 14 and Methods). Oocytes were matured *in vitro*, and cycloheximide was added 30 min before or 60 min after GVBD (Fig. 4a). Extracts were made 2.5 h after GVBD and sperm nuclei were added to follow DNA replication ability. If cycloheximide was added 60 min after GVBD, then DNA replication occurred (Fig. 4b, c), as expected, in a reaction that was totally sensitive to aphidicolin (Fig. 4c). If cycloheximide was added 30 min before GVBD, then Cdc6 was not translated (Fig. 1d) and extracts were incompetent to replicate (Fig. 4b, c). But the addition of recombinant Cdc6 to the extract was sufficient to rescue replication in an aphidicolin-sensitive reaction (Fig. 4c). Furthermore, the simple addition of recombinant Cdc6 to oocyte extracts made at GVBD was sufficient to confer DNA replication competence with an efficiency close to that obtained in egg extracts (Fig. 4d). Finally, the ability to replicate conferred between GVBD and meiosis I to meiosis II transition is inducible by Ca^{2+}, as in the unfertilized egg (Supplementary Information, Fig. 3). These results verify that Cdc6 is the missing DNA replication factor, which starts to be translated at GVBD and is necessary and sufficient to confer to the egg the ability to replicate embryonic DNA after fertilization, when the MPF activity is suppressed. Similar observations were obtained by Coué *et al.*[30] with similar conclusions.

We have identified Cdc6 as the DNA synthesis inducer that has been postulated to give competence to replicate during meiotic maturation before fertilization[2,3]. *cdc6* is expressed as a maternal mRNA in oocytes, and the protein accumulates during progesterone-induced maturation. This regulation occurs at the translational level, similar to the regulation of c-*mos* involved in the CSF activity that maintains the high level of MPF activity necessary for the meiotic arrest of unfertilized eggs[15–18]. The unmasking of specific maternal mRNAs involved in two crucial opposite stages of the cell cycle, initiation of DNA replication and mitosis, therefore contributes to the final stage of oogenesis that leads to the unfertilized egg.

First, competence to form the replication complex is acquired shortly after GVBD with synthesis of Cdc6 (Fig. 5). Cdc6 absence before this last step of oogenesis is likely to provide a way to prevent origin complex formation during the long period of oocyte growth. This acquisition of a licence to replicate at the end of meiosis I might be considered unexpected because preventing DNA replication at this step is crucial to producing a haploid genome. But a negative control of this acquired licence might be achieved by c-*mos*, which starts to accumulate just before GVBD (Fig. 5). The Mos/mitogen-activated protein kinase pathway prevents DNA replication between meiosis I and meiosis II, by prematurely reactivating Cdc2/Cyclin B

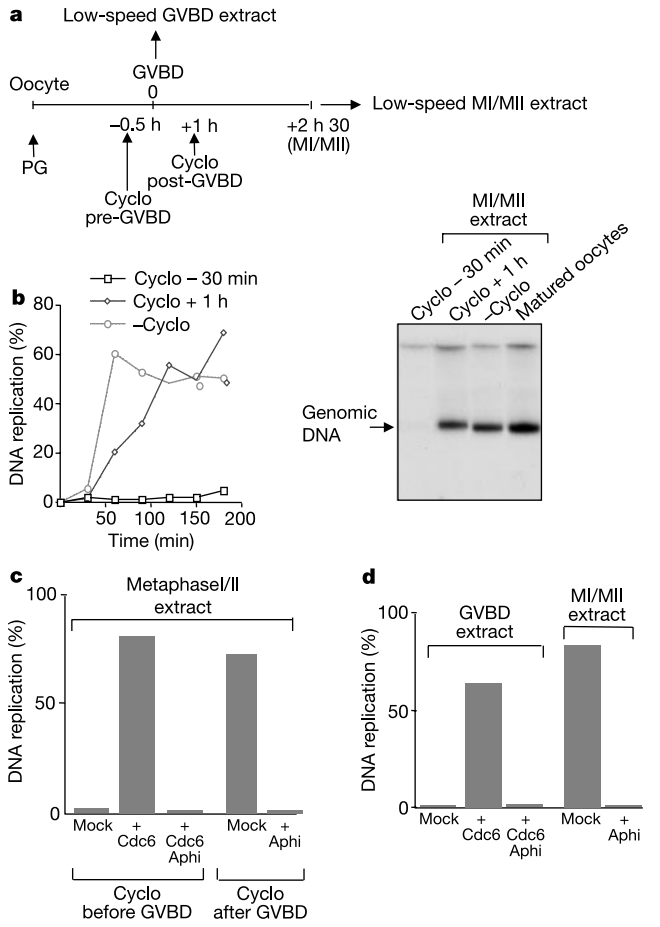

Figure 4 Cdc6 protein is the missing factor acquired soon after GVBD that confers to the egg the competence to replicate. **a**, Stage VI oocytes induced to mature were either left untreated (−cyclo) or treated with 250 μg ml^{-1} cycloheximide 30 min before GVBD (cyclo, −30 min) or 1 h after GVBD (cyclo +1 h). Oocytes were collected either at the GVBD stage, or at the metaphase I/metaphase II (MI/MII) transition (2.5 h after GVBD). Matured oocytes were also collected 12 h after PG addition, and low-speed extracts were prepared. **b**, Sperm nuclei were added to the extracts and DNA synthesis was followed over time and by agarose gel electrophoresis after 2 h. **c**, DNA replication assay was carried out in MI/MII extracts as in **b**. Rescue experiments were carried out by adding purified baculovirus recombinant Cdc6. Aphidicolin (aphi, 100 μg ml^{-1}) inhibited DNA replication at the same concentration as in *Xenopus* egg extracts. **d**, DNA replication assays were carried out in GVBD extracts and Cdc6 rescue experiments were carried out as in **c**.

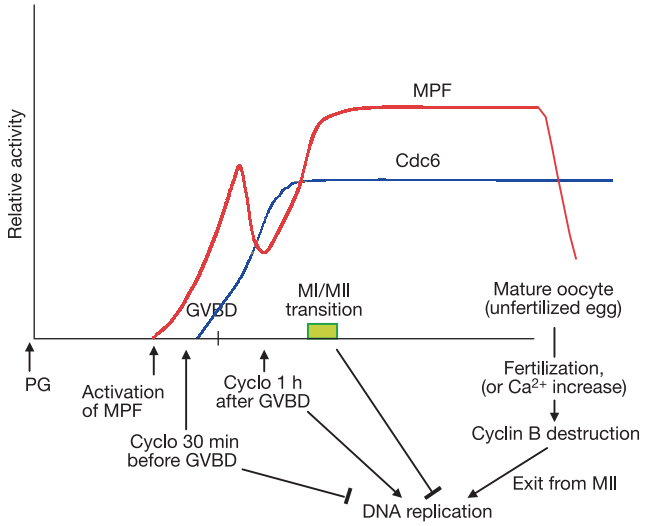

Figure 5 Interplay between replication induced by *cdc6* and its negative control by MPF. Cdc6 is missing in the oocyte but starts to be translated from oocyte *cdc6* maternal mRNA at GVBD during maturation. The second rise of MPF activity prevents activation of DNA replication by Cdc6 during the transition between metaphase I and metaphase II (MI/MII). At fertilization, the degradation of Cyclin B2 and the consequent inactivation of MPF permits the onset of DNA replication in the embryo. Cycloheximide added 30 min before GVBD inhibits DNA replication because it totally prevents the accumulation of Cdc6. Cycloheximide added 1 h after GVBD activates DNA replication because it prevents the second rise of MPF activity when Cdc6 has already started to accumulate.

after metaphase[15–20], thereby allowing meiotic reduction in chromosome number. Consequently, c-*mos* activity during maturation can be considered as a second, superimposed regulation to prevent DNA replication origins firing before fertilization. Geminin, a negative regulator of Cdt1, is present in oocytes as well as in meiosis I/meiosis II extracts from oocytes treated with cycloheximide 1 h after GVBD or from fertilized egg, which are both competent for DNA replication (unpublished data); therefore, its presence cannot explain the repression of DNA replication at these two stages. Emi1, a component of the CSF pathway[21], might be also required in metaphase II arrest. Such coupled regulation, in which the competence to replicate that is given during maturation is repressed until fertilization, represents a way to induce precisely and rapidly the activation of DNA replication immediately after fertilization[22,23].

Our data also emphasize that regulating the recruitment of the MCM helicase to origins is an essential rate-limiting step in initiating DNA replication, and strengthen the role of Cdc6 in determining the replication competence in cells after long periods of quiescence[24,25]. The role of Cdc6 in preparing the egg for fertilization is also emphasized by the observation that Cdc6 can induce ability to bind sperm in immature oocytes[26], a property that is also acquired during maturation. The induction of two apparently unrelated events, competence to replication and sperm-binding ability (which are both essential for fertilization), by the same protein is unexpected. It might unveil links between DNA replication controls and developmental controls, which may be essential in multicellular eukaryotes. □

Methods

Fractionation of oocytes

We prepared defolliculated oocytes as described[14]. Cytoplasmic and germinal vesicle fractions were obtained after manual enucleation either from intact oocytes or from oocytes fixed by 2% trichloroacetic acid (TCA) to prevent leaks from the nucleus. Protein extracts were obtained by grinding oocytes in 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM potassium HEPES, pH 7.7, 50 mM sucrose, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 5 μg ml^{-1} aprotinin, 0.5 mM EGTA and 0.3% Triton X-100, followed by centrifugation for 5 min at 8,000*g*, and were analysed by SDS–PAGE.

Oocyte extracts

Low-speed extracts of maturing oocytes were carried out either at GVBD or 2.5 h after GVBD between meiosis I and meiosis II. GVBD, as judged by the appearance of a white spot at the animal pole, started at between 2 h 45 min and 3.5 h. Maturing oocytes extracts were prepared as described for egg extracts[14] in Eppendorf tubes. After the excess buffer was removed, the oocytes were centrifuged at 8,000*g* for 5 min at 4 °C, and then at 12,000*g* for 2 min at 4 °C. The upper phase was collected and centrifuged again at 12,000*g* for 2 min at 4 °C. We collected the extracts and supplemented them with energy mix[14].

DNA replication assays

We injected oocytes with 25 nl of 5 mCi ml^{-1} [α-^{32}P]dCTP and extracted the DNA 4–5 h after GVBD. Samples with equal numbers of total counts were analysed by 0.8% agarose gel electrophoresis. DNA replication assays were also carried out by quantification of [α-^{32}P]dCTP incorporation in sperm nuclei used at 1,000 nuclei per ml of extract. We carried out analysis by TCA precipitation and migration on 0.8% agarose gels[14]. Purified protein was added at 15 ng per μl of extract to rescue DNA replication activity. We prepared interphasic extract and CSF extract as described[14,27].

Northern blot analysis

Total oocyte RNA was extracted by the LiCl method[28]. We resolved 10 μg of total RNA on a 1% formaldehyde agarose gel and transferred them to Amersham Hybond N + membrane.

Injections

Stage VI oocytes were injected with 25 nl of a 2 mg ml^{-1} solution of a purified *cdc6* 25-nucleotide sense (5′-TCCCCACCCAAGCAGTCTCGCAAAG-3′) or antisense (5′-CTTTGCGAGACTGCTTGGGTGGGGA-3′) oligonucleotide, and induced to mature by addition of progesterone. Control oocytes were injected in parallel with water. For rescue experiments, a 25-nl second injection containing 5 mCi ml^{-1} dCTP and 300 μg ml^{-1} purified Cdc6 protein was injected after GVBD or into matured 12-h oocytes. Maturation was followed by appearance of a clear white spot at the animal hemisphere coupled with manual dissection to check for GVBD.

Cdc6 protein and antibodies

Xenopus His_6–Cdc6 protein was purified from insect cells infected with recombinant baculovirus. We monitored its activity by depletion–rescue experiments as follows: 50 μl of low-speed interphasic extract was either depleted with a specific polyclonal antibody to Cdc6 or mock-depleted with pre-immune serum immunoglobulin-γ, as described[29]. We added purified Cdc6 protein to the depleted extract and measured DNA replication (Supplementary Information Fig. 1). The rabbit polyclonal antibody against Cdc6 and antibodies against Cdt1, MCM4, MCM3, RPA34 and Geminin were obtained by four injections of corresponding recombinant proteins. Other antibodies were generous gifts from J. Blow (ORC1), H. Takisawa (Cdc45, MCM2, MCM5, MCM6 and MCM7), J. Maller (Cdc7), Y. Adachi (RPA70) and J. Walter (ORC2). The *Xenopus* Cdc6 baculovirus[6] was a generous gift from T. Coleman.

Received 9 May; accepted 10 July 2002; doi:10.1038/nature01046.

1. Masui, Y. & Clarke, H. J. Oocyte maturation. *Int. Rev. Cytol.* **57,** 185–282 (1979).
2. Gurdon, J. B. On the origin and persistence of a cytoplasmic state inducing nuclear DNA synthesis in frogs eggs. *Proc. Natl Acad. Sci. USA* **58,** 545–552 (1967).
3. Furuno, N. *et al.* Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.* **13,** 2399–2410 (1994).
4. Kelly, T. J. & Brown, G. W. Regulation of chromosome replication. *Annu. Rev. Biochem.* **69,** 829–880 (2000).
5. Lei, M. & Tye, B. K. Initiating DNA synthesis: from recruiting to activating the MCM complex. *J. Cell Sci.* **114,** 1447–1454 (2001).
6. Coleman, T. R., Carpenter, P. B. & Dunphy, G. The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87,** 53–63 (1996).
7. Pelizon, C., Madine, M. A., Romanowski, P. & Laskey, R. A. Unphosphorylatable mutants of Cdc6 disrupt its nuclear export but still support DNA replication once per cell cycle. *Genes Dev.* **14,** 2526–2533 (2000).
8. Okuno, Y., McNairn, A. J., den Elzen, N., Pines, J. & Gilbert, D. M. Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. *EMBO J.* **20,** 4263–4277 (2001).
9. Coue, M., Kearsey, S. E. & Mechali, M. Chromatin binding, nuclear localization and phosphorylation of *Xenopus* Cdc21 are cell-cycle dependent and associated with the control of initiation of DNA replication. *EMBO J.* **15,** 1085–1097 (1996).
10. Pereverzeva, I., Whitmire, E., Khan, B. & Coue, M. Distinct phosphoisoforms of the *Xenopus* Mcm4 protein regulate the function of the Mcm complex. *Mol. Cell. Biol.* **20,** 3667–3676 (2000).
11. Hara, K., Tydeman, P. & Kirschner, M. A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc. Natl Acad. Sci. USA* **77,** 462–466 (1980).
12. Harland, R. M. & Laskey, R. A. Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. *Cell* **21,** 761–771 (1980).
13. Blow, J. J. & Laskey, R. A. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* **47,** 577–587 (1986).
14. Menut, S., Lemaitre, J. M., Hair, A. & Méchali, M. in *Advances in Molecular Biology: a Comparative Methods Approach to the Study of Ooocytes and Embryos* (ed. Richter, J. D.) 196–226 (Oxford Univ. Press, Oxford, 1999).
15. Sagata, N., Watanabe, N., Vande Woude, G. F. & Ikawa, Y. The c-*mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342,** 512–518 (1989).
16. Yew, N., Mellini, M. L. & Vande Woude, G. F. Meiotic initiation by the mos protein in *Xenopus*. *Nature* **355,** 649–652 (1992).
17. Nebreda, A. R., Gannon, J. V. & Hunt, T. Newly synthesized protein(s) must associate with p34cdc2 to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. *EMBO J.* **14,** 5597–5607 (1995).
18. Hochegger, H. *et al.* New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation. *Development* **128,** 3795–3807 (2001).
19. Abrieu, A., Doree, M. & Fisher, D. The interplay between cyclin-B–Cdc2 kinase (MPF) and MAP kinase during maturation of oocytes. *J. Cell Sci.* **114,** 257–267 (2001).
20. Masui, Y. The elusive cytostatic factor in the animal egg. *Nature Rev. Mol. Cell. Biol.* **1,** 228–232 (2000).
21. Reimann, J. D. & Jackson, P. K. Emi1 is required for cytostatic factor arrest in vertebrate eggs. *Nature* **416,** 850–854 (2002).
22. Weinreich, M., Liang, C., Chen, H. H. & Stillman, B. Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle. *Proc. Natl Acad. Sci. USA* **98,** 11211–11217 (2001).
23. Calzada, A., Sacristan, M., Sanchez, E. & Bueno, A. Cdc6 cooperates with Sic1 and Hct1 to inactivate mitotic cyclin-dependent kinases. *Nature* **412,** 355–358 (2001).
24. Madine, M. A. *et al.* The roles of the MCM, ORC, and Cdc6 proteins in determining the replication competence of chromatin in quiescent cells. *J. Struct. Biol.* **129,** 198–210 (2000).
25. Mendez, J. & Stillman, B. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol. Cell. Biol.* **20,** 8602–8612 (2000).
26. Tian, J., Thomsen, G. H., Gong, H. & Lennarz, W. J. *Xenopus* Cdc6 confers sperm binding competence to oocytes without inducing their maturation. *Proc. Natl Acad. Sci. USA* **94,** 10729–10734 (1997).
27. Lemaitre, J. M., Geraud, G. & Mechali, M. Dynamics of the genome during early *Xenopus laevis* development: karyomeres as independent units of replication. *J. Cell Biol.* **142,** 1159–1166 (1998).
28. Taylor, M. V., Gusse, M., Evan, G. I., Dathan, N. & Mechali, M. *Xenopus myc* proto-oncogene during development: expression as a stable maternal mRNA uncoupled from cell division. *EMBO J.* **5,** 3563–3570 (1986).
29. Maiorano, D., Moreau, J. & Mechali, M. XCDT1 is required for the assembly of pre-replicative complexes in *Xenopus laevis*. *Nature* **404,** 622–625 (2000).
30. Whitmire, E., Khan, B. & Coué, M. Cdc6 synthesis regulates replication competence in *Xenopus* oocytes. *Nature* **419,** 722–725 (2002).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com/nature).

Acknowledgements We thank P. Françon for help; N. Montel for technical assistance; and B. Honda, D. Maiorano, D. Fisher and C. Jaulin for critically reading the manuscript. This work has been supported by grants from the Association pour la Recherche sur le Cancer and the Human Frontier Science Program.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.M. (e-mail: mechali@igh.cnrs.fr).

Cdc6 synthesis regulates replication competence in *Xenopus* oocytes

Elizabeth Whitmire, Bettina Khan & Martine Coué

Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, 3601 4th Street, Lubbock, Texas 79430, USA

The early division cycles of an embryo rely on the oocyte's ability to replicate DNA. During meiosis, oocytes temporarily lose this ability. After a single round of pre-meiotic S-phase, oocytes enter meiosis and rapidly arrest at prophase of meiosis I (G2)[1]. Upon hormonal stimulation, arrested oocytes resume meiosis, re-establish DNA replication competence in meiosis I shortly after germinal vesicle breakdown (GVBD), but repress replication until fertilization[2,3]. How oocytes lose and regain replication competence during meiosis are important questions underlying the production of functional gametes. Here we show that the inability of immature *Xenopus* oocytes to replicate is linked to the absence of the Cdc6 protein and the cytoplasmic localization of other initiation proteins. Injection of Cdc6 protein into immature oocytes does not induce DNA replication. However, injection of Cdc6 into oocytes undergoing GVBD is sufficient to induce DNA replication in the absence of protein synthesis. Our results show that GVBD and Cdc6 synthesis are the only events that limit the establishment of the oocyte's replication competence during meiosis.

The focus of our study is the acquisition of replication competence of *Xenopus* oocytes during meiosis, a question that was first examined by Gurdon in 1967[1]. Subsequently, the replication defect of immature *Xenopus* oocytes arrested at prophase of meiosis I was linked to the initiation step rather than the elongation step of DNA replication[4]. *Xenopus* immature oocytes contain functional polymerases that can support semi-conservative replication of single-stranded DNA[5]. Initiation of eukaryotic DNA replication requires the assembly onto the chromatin of pre-replication complexes (pre-RCs) containing Orc, Cdc6, Cdt1 and Mcm proteins[6,7]. The pre-RCs are then activated by the Cdk2/cyclin E and Cdc7/Dbf4 kinases leading to binding of the Cdc45 protein to the pre-RCs and unwinding of the DNA[8,9]. To determine whether the initiation defect corresponds to a defect in pre-RC assembly or activation, we looked at the expression of pre-RC components and activating kinases in immature (stage VI) *Xenopus* oocytes. Western blot analysis showed that all but one of the initiation factors tested

