The Cdc7 kinase is essential for the initiation of DNA replication in eukaryotes. Two regulatory subunits of the Xenopus Cdc7 kinase have been identified: XDbf4 and XDrf1. In this study we determined the expression pattern of XDbf4 and XDrf1 and examined their involvement in DNA replication. We show that XDrf1 expression is restricted to oogenesis and early embryos, whereas XDbf4 is expressed throughout development. Immunodepletion from Xenopus egg extracts indicated that both proteins are only found in complexes with XCdc7 and there is a 5-fold molar excess of the XCdc7/Drf1 over XCdc7/Dbf4 complexes. Both complexes exhibit kinase activity and are differentially phosphorylated during the cell cycle. Depletion of the XCdc7/Drf1 from egg extracts inhibited DNA replication, whereas depletion of XCdc7/Dbf4 had little effect. Chromatin binding studies indicated that XCdc7/Drf1 is required for pre-replication complex activation but not their assembly. XCdc7/Dbf4 complexes bound to the chromatin in two steps: the first step was independent of pre-replication complex assembly and the second step was dependent on pre-replication complex activation. By contrast, binding of XCdc7/Drf1 complexes was entirely dependent on pre-replication complex assembly. Finally, we present evidence that the association of the two complexes on the chromatin is not regulated by ATR checkpoint pathways that result from DNA replication blocks. These data suggest that Cdc7/Drf1 but not Cdc7/Dbf4 complexes support the initiation of DNA replication in Xenopus egg extracts and during early embryonic development.

In oocytes, initiation of DNA replication requires the assembly and activation of pre-replication complexes (pre-RCs) on chromatin (1). Sequential binding to DNA of the origin recognition complex, Cdc6, Cdt1, and mini-chromosome maintenance proteins (Mcm2–7) lead to formation of pre-RCs. Pre-RC activation is under the control of two kinases, Cdk2 and Cdc7, and ultimately results in the loading of replication factors such as Cdc45 and the unwinding of replication origins by the MCM helicase complex (2–5).

Cdc7 is a serine/threonine kinase that is conserved from yeast to human and is essential for cell proliferation and embryonic development (6). Like CDKs (cyclin-dependent kinases), Cdc7 activity is regulated by its association with a regulatory subunit, the Dbf4 protein. This complex is often referred to as DDK (Dbf4-dependent kinase). The existence in fission yeast of a Cdc7/Dbf4 complex paralog, Spo4/Spo6, and the recent discovery of multiple Dbf4-related molecules in animal cells suggest that they belong to a novel DDK protein kinase family (7–10). Functional differences between family members have started to emerge but they need to be further characterized. In fission yeast the Spo4/Spo6 kinase complex is required for meiosis, whereas the other DDK, Hsk1/Dfp1, not only regulates initiation of DNA replication but also centromere cohesion mediated by heterochromatin (11, 12). Two DDKs have recently been identified in humans and Xenopus laevis, the Cdc7/Dbf4 and Cdc7/Drf1 complexes (9, 13, 14). Whereas both kinase complexes are able to phosphorylate the Mcm2 protein in vitro, the Cdc7/Dbf4 complex is believed to be the one essential for DNA replication (10, 13, 14). On the other hand, the Cdc7/Drf1 complex appears to be required for efficient progression through S and M phases in human but is dispensable for initiation of DNA replication in the cell-free system derived from Xenopus eggs (9, 10). In addition, both Xenopus complexes seem to play a role in checkpoint pathways. The Cdc7/Drf1 complex appears to suppress Cdc45 chromatin binding in an ATR-dependent manner during replication block and an ATR-dependent DNA damage checkpoint inhibits Cdc7/Dbf4 kinase activity by disassociating the complex (9, 15).

Our independent identification of the Xenopus Dbf4 and Drf1 regulatory subunits led us to compare side by side their developmental expression and their requirement for the initiation of DNA replication in Xenopus egg extracts. Our results indicate that XDrf1 is present in early embryos, whereas XDbf4 is present throughout development. In Xenopus egg extracts both regulatory subunits are only found in complexes with the Cdc7 kinase and there is a 5-fold molar excess of the Cdc7/Drf1 over Cdc7/Dbf4 complexes. Both complexes exhibit kinase activity and phosphorylate Mcm2 in vitro. However, contrary to what was previously reported, initiation of DNA replication in Xenopus egg extracts requires predominantly Cdc7/Drf1 and not Cdc7/Dbf4.

**EXPERIMENTAL PROCEDURES**

Isolation of Xenopus Oocytes and Embryos—Oocytes were obtained from Xenopus ovary fragments treated with 2 mg/ml collagenase to remove follicle cells. Oocytes I–VI were staged manually following the Dumont classification (16). To induce maturation, stage VI oocytes were incubated in the presence of 10 μg/ml progesterone. Xenopus eggs were fertilized in vitro, dejellied in 2% cysteine, 0.1 mM MMR (0.5 mM HEPES, pH 7.8, 10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO4, 0.2 mM CaCl2, 0.01 mM EDTA), and maintained in 0.1× MMR. Embryos were staged according to Nieuwkoop (17). Embryos were collected at the times indicated, snap frozen on dry ice, and stored at −80 °C.

Northern and Western Blot Analysis during Development—Total RNA was isolated from oocytes and embryos by using Trizol reagent...
Xcdc7/Drf1 Required for DNA Replication

(V)Invitrogen) followed by phenol-chloroform extraction and isooamyl alcohol precipitation. Fifteen micrograms of RNA were resolved by denaturing gel electrophoresis, transferred to a nylon membrane, and probed with radiolabeled full-length Xcdc7 or XDbf4 probes. A fragment of the Xdrf1 gene corresponding to the C-terminal half of the protein (amino acids 291–784) was used as a probe. Ethidium bromide-stained 18 S and 28 S rRNA were used as loading controls, indicating that comparable amounts of RNA were loaded in all lanes. For Western blot analysis, 20 oocytes or embryos were homogenized in 200 µl of extract buffer (20 mM K-HEPES, pH 7.8, 100 mM KCl, 5 mM MgCl2, 1 mM EGTA, 50 mM sucrose, 0.2% Triton, and 5 µg/ml aprotinin, leupetin, and pepstatin). Extracts were centrifuged at 4 °C for 10 min at 20,000 × g. As indicated, extracts were treated with 0.17 units/µl of shrimp alkaline phosphatase for at least 30 min at 37 °C. Extract equivalent to 3 oocytes or embryos was analyzed by SDS-PAGE.

Recombinant Protein Production—Xcdc7/Drf1 and Xcdc7/XDbf4 complexes were expressed in bacteria using the pETDuet-1 bicistronic vector from Novagen. The following sets of primers were used for PCR amplification: Xcdc7, 5'-GACGGCCATATGCTTGCTCCAGGCACTTGGATCATGGGTCAGAATGTTGC-3' and XDbf4, 5'-GAGAACATTGGAGTACATGACCCACCA-TCACCATCTAAATCTACCATAGCAGC-3'. Xenopus, 5'-GGGTTGATCCCTACCGCATGTTTTTAAAC-3' and 5'-GAGGAAGATCTCAACATTGCCTGACGAC-3'. Xdrf1, 5'-GGATCCCTACCGCATGTTTTTAAACA-3' and 5'-GGGAACATTGGAGTACATGACCCACCA-TCACCATCTAAATCTACCATAGCAGC-3'. The cloning sites used were NdeI/BglII for Xcdc7, NcoI/BamHI for XDrf1, and EcoRI/NotI for XDbf4.

Antibodies—Polyclonal antibodies against XenoD cdc6, Cdt1, Mcm4, Cdc7, XDbf4, and Cdc45 were raised in rabbits using Escherichia coli His-tag recombinant full-length proteins as antigens (18, 19). A C-terminal portion of the Xenopus Drf1 protein (amino acids 291–784) was expressed with a His tag in E. coli and used as antigen. Rabbit sera were affinity purified by column chromatography against immobilized proteins as previously described (20).

Extract Preparation and Immunodepletion—Interphase and mitotic arrested egg extracts were prepared according to Murray (21) with the exception that interphase extracts contained 0.25 mg/ml cycloheximide. All extracts were supplemented with 3% glycerol, aliquoted, and stored at −80 °C. For depletion, 0.5 volumes of antibody-bound protein A-Sepharose (Amersham Biosciences) was incubated with extracts for 1 h at 4 °C. Depleted extract and beads were separated by centrifugation (5 s at 1000 × g) through a cut-down 200-µl pipette tip containing a glass bead at the bottom. One to two microliters of extract were used for Western blot analysis. As a result of the depletion procedure we found that the concentration of the Cdc7, XDbf4, and Drf1 proteins in the mock depleted extract was about 20–25% lower than in a non-depleted extract due to dilution and/or nonspecific protein absorption to the beads during the procedure. TrueBlot anti-rabbit secondary antibody (eBioscience) was used for the detection of the proteins bound to the beads to reduce the background signal (Fig. 48).

Replication Assay, Nuclei, and Chromatin Isolation—Demembranated Xenopus sperm nuclei were prepared as previously described (22). Nuclei were incubated in interphase egg extracts for the indicated times at 23 °C and at a concentration of 2500 sperm heads/µl of extract unless specified otherwise in the figure legend. To monitor DNA replication ([α-32P]dCTP was also added to extracts and after a 90-min incubation, 1 volume of stop buffer (80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.1% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% bromphenol blue, and 1.0 mg/ml proteinase K) was added to the reaction. After 2 h of incubation at 37 °C, the sample was run on a 0.8% agarose gel and subsequently analyzed with a PhosphorImager instrument (Amersham Biosciences). Alternatively, DNA synthesis was measured by [α-32P]dCTP incorporation into acid-insoluble material as described (23). Nuclei and chromatin isolation was carried out as previously described (18).

Kinase Assays—Xcdc7 complexes were depleted from 20 µl of extract by using Xdc7, Drf1, or Dxbf4 antibodies coupled to protein A-Sepharose. Beads were then washed several times with kinase buffer (40 mM HEPES, pH 7.4, 100 mM sucrose, 100 mM KCl, 15 mM MgCl2, 0.1% Nonident P-10, 40 mM NaF, 80 mM β-glycerophosphate, 1 mM dithiothreitol, and 10 µM ATP) and incubated with 5 µCi of [γ-32P]ATP and 4 µg of purified Xmc2 protein in kinase buffer for 30 min at 23 °C. Reactions were stopped by adding SDS-PAGE sample buffer, subjected to electrophoresis, and autoradiography. H1 kinase assay was performed as previously described (19).

Phosphorylation Assays—35S-Labeled XDrf1 and XDbf4 proteins were translated in vitro by using a TnT Coupled Reticulocyte Lysate System from Promega. Labeled proteins (0.5 µl) were incubated in 10-µl egg extracts in the presence or absence of kinase inhibitor or alkaline phosphatase for 60 min at 23 °C. Proteins in the extract were then separated by SDS-PAGE and the phosphorylation-induced shift of the labeled proteins was determined by phosphoimager analysis. An anti-human phospho-Chk1 Ser^345 antibody from Cell Signaling Technology was used to detect the ATR-dependent phosphorylation of the Chk1 protein.

RESULTS

Identification of the Xenopus Dbf4 and Drf1 Subunits—By searching the GenBank™ data base for a potential Xenopus homolog of the human Dbf4 protein we identified two expressed tag sequences (clone image 3402603 and 3405554). Sequencing of these clones revealed that each contains a complete reading frame. The first one (3402603) is identical to the XDbf4 cDNA previously reported in the literature (Refs. 13 and 14, and GenBank accession numbers AB095983 and AF448801 and CR942472). Alignments of the Xenopus and human Dbf4 and Drf1 proteins indicated that the percent identity between these proteins is low overall but increases over the N-terminal half of the proteins where the N, M, and C conserved motifs are found (24) (Fig. 1A). The C-terminal of the Xenopus Drf1 protein is significantly longer and surprisingly shares the least similarity with the C-terminal of the human Drf1 homolog. Rabbit polyclonal antibodies were raised against the C-terminal portion of the XDrf1 protein or the full-length XDbf4 protein and used as antigens (18, 19). A second clone (3405554) encodes a polypeptide that is identical to human Dbf4, and surprisingly shares the least similarity with the C-terminal of the XDbf4 protein as the clone (3402603) does (Ref. 9, and GenBank accession number AF448801 and CR942472). The cloning sites used were NdeI/BglII for Xcdc7, NcoI/BamHI for XDbf4, and EcoRI/NotI for XDbf4. BL21 DE3 cells were transformed with the pETDuet-Xcdc7/XDbf4 or pETDuet-Xdrf1 constructs and induced with 0.2 mM isopropyl 1-thio-galactosynoside for 24 h at 23 °C to maximize the expression of soluble complexes. Cells were resuspended in lysis buffer (20 mM NaH2PO4, pH 8.0, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100) containing 1 mg/ml lysozyme and then sonicated. After centrifugation at 40,000 × g for 30 min at 4 °C, the supernatant (eBioscience) was used for the detection of the proteins bound to the beads to reduce the background signal (Fig. 48).

Replication Assay, Nuclei, and Chromatin Isolation—Demembranated Xenopus sperm nuclei were prepared as previously described (22). Nuclei were incubated in interphase egg extracts for the indicated times at 23 °C and at a concentration of 2500 sperm heads/µl of extract unless specified otherwise in the figure legend. To monitor DNA replication ([α-32P]dCTP was also added to extracts and after a 90-min incubation, 1 volume of stop buffer (80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.1% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% bromphenol blue, and 1.0 mg/ml proteinase K) was added to the reaction. After 2 h of incubation at 37 °C, the sample was run on a 0.8% agarose gel and subsequently analyzed with a PhosphorImager instrument (Amersham Biosciences). Alternatively, DNA synthesis was measured by [α-32P]dCTP incorporation into acid-insoluble material as described (23). Nuclei and chromatin isolation was carried out as previously described (18).

Kinase Assays—Xcdc7 complexes were depleted from 20 µl of extract by using Xdc7, Drf1, or Dxbf4 antibodies coupled to protein A-Sepharose. Beads were then washed several times with kinase buffer (40 mM HEPES, pH 7.4, 100 mM sucrose, 100 mM KCl, 15 mM MgCl2, 0.1% Nonident P-10, 40 mM NaF, 80 mM β-glycerophosphate, 1 mM dithiothreitol, and 10 µM ATP) and incubated with 5 µCi of [γ-32P]ATP and 4 µg of purified XMc2 protein in kinase buffer for 30 min at 23 °C. Reactions were stopped by adding SDS-PAGE sample buffer, subjected to electrophoresis, and autoradiography. H1 kinase assay was performed as previously described (19).

Phosphorylation Assays—35S-Labeled XDrf1 and XDbf4 proteins were translated in vitro by using a TnT Coupled Reticulocyte Lysate System from Promega. Labeled proteins (0.5 µl) were incubated in 10-µl egg extracts in the presence or absence of kinase inhibitor or alkaline phosphatase for 60 min at 23 °C. Proteins in the extract were then separated by SDS-PAGE and the phosphorylation-induced shift of the labeled proteins was determined by phosphoimager analysis. An anti-human phospho-Chk1 Ser^345 antibody from Cell Signaling Technology was used to detect the ATR-dependent phosphorylation of the Chk1 protein.
protein. Both XDrf1 and XDbf4 preimmune sera showed no cross-reactivity with interphase (LS) or mitotic (CSF) Xenopus egg extracts (data not shown). After affinity purification, each antibody specifically recognized recombinant XDbf4 or XDrf1 proteins translated in vitro using reticulocyte lysate (Fig. 1B). By Western blot analysis, our XDrf1 antibody detected one major polypeptide of about 145 kDa in LS or CSF Xenopus egg extracts (Fig. 1C). Phosphatase treatment of the endogenous XDrf1 increased its electrophoretic mobility indicating that the protein is phosphorylated in both LS and CSF extracts. Detection of the endogenous XDbf4 protein in egg extracts was more difficult as it migrated as a smear in the SDS-PAGE gel. However, a well defined electrophoretic band of 73 kDa corresponding to XDbf4 was obtained after phosphatase treatment (Fig. 1C). Similar electrophoretic behavior of XDbf4 was also observed by Furukori et al. (13). Using Western blot analysis and recombinant XDbf4 and XDrf1 as standards, we estimated the concentration of these two proteins in Xenopus egg extract to be 10 nM for XDbf4 and 50 nM for XDrf1 (data not shown). The concentration of the XCdc7 protein in the same extract was previously estimated to be 60 nM (13).

Developmental Expression of XDbf4 and XDrf1—Northern and Western blot analyses were used to determine the developmental expression of the Xenopus Dbf4, Drf1, and Cdc7 genes (Fig. 2). We found that the three transcripts were present during oogenesis (stages I–VI) and early embryogenesis (Fig. 2A). After the onset of zygotic transcription (stage 8.5) the amount of XDrf1 and XCdc7 mRNAs declined abruptly to undetectable levels. Longer exposure of the Northern blots revealed the presence of a very small amount of the XCdc7 message (but not XDrf1) in late stages of embryogenesis and in cultured A6 cells derived from Xenopus kidney. On the other hand, the XDbf4 mRNA was found throughout development and in A6 cells. Protein expression profiles were also followed during oogenesis and maturation (Fig. 2B) as well as embryonic development (Fig. 2C). Low levels of XCdc7 and XDrf1 proteins were detected in growing oocyte stages III–VI and V–VI, respectively (Fig. 2B). Expression of both proteins increased significantly during oocyte maturation. No XDbf4 protein was found in oocytes I–VI but it was detected at maturation (Fig. 2B). After fertilization, the level of the XDrf1 protein decreased slowly until the mid-blastula transition (MBT, stage 8.5). A sharper decrease occurred after MBT correlating with the disappearance of the XDrf1 message (Fig. 2C). No XDrf1 protein was detected in embryos past stage 10 of development. The level of XCdc7 protein also decreased during embryonic development but at a slower rate than the XDrf1 protein. Interestingly, low levels of the XCdc7 protein were still found in late embryos where the mRNA is barely detectable. A similar pattern of Cdc7 expression in Xenopus embryos was previously reported by Roberts et al. (25). Finally, the level of XDbf4 protein remained constant from mature oocytes to stage 8.5 embryos but increased after MBT (Fig. 2C). Together, these results suggest that the Xenopus Cdc7 and Drf1 characterized in this study are the products of maternal genes whose expression is restricted to early stages of development. Whereas we have no evidence supporting the existence of a zygotic homolog for XDrf1, our data suggest that a zygotic homolog or spliced variant of the XCdc7 gene accounts for the expression of the Cdc7 protein in late embryos and somatic cells. On the other hand, only one Xenopus Dbf4 homolog seems to be expressed throughout development.

XDbf4 and XDrf1 Are Differentially Phosphorylated during the Cell Cycle—Results described above (Fig. 1C) indicate that both XDbf4 and XDrf1 are phosphorylated in interphase and mitotic Xenopus egg
extracts. CDKs are known to phosphorylate a number of replication factors in Xenopus and autophosphorylation is one characteristic of the yeast and human DDKs (6). Therefore we tested the involvement of these two kinases in the phosphorylation of XDbf4 and XDrf1 in interphase and mitotic egg extract (Fig. 3). Our inability to identify discrete phosphoisoforms of the endogenous proteins by Western blot analysis led us to follow the phosphorylation of 35S-labeled recombinant XDbf4 and XDrf1 added to egg extracts. After incubation in interphase extract both labeled proteins exhibited a slower electrophoretic migration similar to the endogenous proteins (compare Fig. 3A with Fig. 1C). Addition of 6′-DMP, a S/T kinase inhibitor, or phosphatase to the extract prevented the shift in mobility of both proteins confirming that it reflects their phosphorylation status (Fig. 3A). Phosphorylation of XDbf4 and XDrf1 in interphase extracts was not affected by the presence of two CDK inhibitors (rosocvitine and p21) but did not occur in a Cdc7-depleted extract (Fig. 3A). Controls for the inhibition of CDK activity by roscovitine in CSF extract and Cdc7 depletion from both LS and CSF Xenopus egg extracts are presented in supplemental Fig. S1. Whereas roscovitine did not affect XDbf4 phosphorylation in LS extracts, we consistently observed a partial degradation of the 35S-labeled recombinant XDbf4 (Fig. 3A). However, such degradation did not occur in the presence of p21 (Fig. 3A), or in CSF extracts (Fig. 3B). The reason for this differential stability of Dbf4 is currently unknown. Both XDbf4 and XDrf1 proteins also exhibited mobility shifts when incubated in mitotic egg extracts (CSF for cytostatic factor-arrested extract). The shift of the XDbf4 protein was larger in mitotic than interphase extracts (compare lanes 2 and 4, Fig. 3B) suggesting that more sites might be phosphorylated during mitosis. Cdc7 depletion from mitotic extracts reduced the extent of the shift but its complete suppression required both Cdc7 depletion and CDK inhibitors. The shift induced by the phosphorylation of XDrf1 was identical in interphase and mitotic extract and depended only on Cdc7 (Fig. 3B). Overall these results indicate that the phosphorylation of XDbf4 varies during the cell cycle and requires Cdc7 and CDKs during mitosis but only Cdc7 in interphase. XDrf1 phosphorylation appears to be stable throughout the cell cycle requiring only Xcdc7.

**XDbf4 and XDrf1 Are Only Found in Active Complexes with Xcδ7 in Xenopus Egg Extracts**—The coexistence of two regulatory subunits of the Cdc7 kinase in egg extracts prompted us to examine their association with Xcdc7. Antibodies against Xcdc7, XDrf1, and XDbf4 were used to deplete interphase egg extracts. The depleted extracts, as well as the proteins bound to specific IgG beads were analyzed by Western blot (Fig. 4). The Xcdc7 antibodies completely depleted Xcdc7 as well as XDrf1 and XDbf4 proteins from the extracts (Fig. 4A). These three proteins were also found on Xcdc7 beads (Fig. 4B). Depletion of XDrf1 resulted in the depletion of about 85% of Xcdc7 present in the extract but did not affect the XDbf4 level. Alternatively, a small amount of Xcdc7 (around 10%) was co-depleted by XDbf4 antibodies (Fig. 4A). These results confirm the interaction of the two regulatory subunits with Cdc7 in Xenopus egg extracts (9, 13, 14). Furthermore, quantitative depletions establish that both subunits are only found in complexes with Xcdc7. Contrary to previous reports, no free XDrf1 or XDbf4 were found in the extracts (9, 13). Taking into account these results and the concentration of these proteins in interphase egg extracts, we estimated that there is five times more Xcdc7/Drf1 than Xcdc7/Dbf4 complexes in interphase extracts with concentrations of 50 and 10 mM, respectively. Finally, we noticed that the depletion of one complex did not affect the phosphorylation of the regulatory subunit in the other complex (compare +/− shrimp alkaline phosphatase (SAP) in Fig. 4A). This indicates that the Cdc7-dependent phosphorylation of the XDrf1 and XDbf4 subunits we previously observed in interphase extracts is the result of auto-phosphorylation events.

Next, we determined and compared the kinase activity of the two complexes obtained by immunodepletion of interphase egg extract with antibodies against XDbf4 and XDrf1 (Fig. 5A). Each complex bound to protein A-IgG beads was able to phosphorylate a recombinant Mcm2 protein in vitro but the kinase activity associated to Xcdc7/Drf1 was approximately five times that of Xcdc7/Dbf4. This difference in kinase activity correlated with the amount of each complex in the extract or on the beads and therefore did not appear to reflect a difference in the specific activity of the complexes.
FIGURE 5. Both Xenopus Cdc7 complexes have kinase activities but only XCdc7/Drf1 is required for DNA replication. A, XCdc7/Dbf4 and XCdc7/Drf1 complexes were obtained by immunodepleting interphase egg extracts with antibodies against XDbf4 or XDrf1. Complexes were then incubated in the presence of baculovirus recombinant XMcm2 protein and [γ-32P]ATP for in vitro kinase assay. Phosphorylation of Mcm2 was followed by SDS-PAGE and phosphoimager analysis. B, sperm nuclei (2500/μl) were incubated in extracts depleted with XCdc7, XDrf1, XDbf4, or non-immune antibodies and in the presence of [γ-32P]dCTP for 90 min. Products of the replication reactions were separated on an agarose gel and visualized by autoradiography. C, increasing amounts of XCdc7/Drf1 or XCdc7/Dbf4 recombinant complexes were added to Cdc7-depleted LS extracts containing sperm nuclei (2500/μl) and [γ-32P]dCTP. DNA replication was determined by measuring the maximum [γ-32P]dCTP incorporation into acid-insoluble material that occurred at 180 min. 100% replication was achieved in mock depleted extracts containing recombinant complexes or buffer. The values under the bar graph are the concentrations of the added complexes expressed in nanomolar and also in molar equivalents of endogenous complexes in the mock depleted extract.

XCdc7/Drf1 but Not XCdc7/Dbf4 Is Required for Initiation of DNA Replication in Xenopus Egg Extracts—Depletion of Cdc7 from Xenopus egg extracts has been shown to inhibit both the DNA replication and the binding of Cdc45 to chromatin (3, 4). To determine whether only one or both Cdc7 complexes play a role in DNA replication, we tested the effect of their specific depletion on the ability of Xenopus egg extracts to replicate sperm chromatin (Fig. 5B and supplemental Fig. S2A for depletion control). Extracts were depleted with anti-XDrf1 antibodies or mock depleted with non-immune antibodies as a control. As expected, Cdc7-depleted extracts supported very little DNA replication in 90 min when compared with mock depleted extracts. Depletion of XDrf1 also strongly inhibited sperm chromatin replication, whereas XDbf4 depletion had little effect (Fig. 5B). Although the amount of DNA replication observed in XDrf1-depleted extracts was low, it was consistently higher than in XCdc7-depleted extracts. This suggested that the XCdc7/Dbf4 complexes present in Xenopus extracts can support replication but its low efficiency may be related to its low concentration in extracts compared with XCdc7/Drf1. Overall, these results indicate that among the two Cdc7 complexes present in egg extracts XCdc7/Drf1 is the one supporting DNA replication. Our finding disagrees with a report from Yanow et al. (9) that concluded that XDrf1 is not required for DNA replication in egg extracts. The basis for this disagreement is unclear but may be related to the efficiency with which extracts were depleted. To further verify that XCdc7/Drf1 supports replication, we prepared bacterial recombinant XCdc7/Drf1 and XCdc7/Dbf4 complexes to test their ability to restore DNA replication to Cdc7-depleted extracts that contained neither of the two complexes. Both recombinant complexes were active as they did phosphorylate a recombinant Mcm2 protein in vitro and showed signs of autophosphorylation (supplemental Fig. S2B). Titration experiments showed that either complex rescued DNA replication in Cdc7-depleted extracts (Fig. 5C). Complete rescue was achieved by the addition of 38 nM XCdc7/Drf1 complex, which corresponds to the concentration of this complex in the control mock-depleted extract. Higher amounts of XCdc7/Drf1 led to no further increase, suggesting that no re-replication occurred (data not shown). On the other hand, partial DNA replication rescue was also obtained with recombinant XCdc7/Dbf4 complex. Full rescue was not possible due to the dilute concentration of the purified XCdc7/Dbf4; thus, at the maximal volume of this complex for this assay (10% of the extract volume), rescue to 80% of DNA replication in the control was obtained with 70 nM XCdc7/Dbf4, which represents a 9-fold molar excess over the mock-depleted extract. The XCdc7/Drf1 complex was more efficient than the XCdc7/Dbf4 complex at rescuing replication. For example, 50% rescue was obtained with 4.9 nM XCdc7/Drf1 versus 57.5 nM for XCdc7/Dbf4. Finally, it is interesting to note that the depletion of Cdc7 from the extract did not completely abolish DNA replication. In the two experiments presented in Fig. 5C, the maximum level of DNA replication in Cdc7-depleted extracts was attained by 180 min and reached 43 and 50% of the mock depleted extracts, respectively. These findings suggest that while the presence of the XCdc7/Drf1 complexes in Xenopus egg extracts is required for efficient and complete DNA replication, other mechanisms independent of Cdc7 complexes can support a limited replication process. Evidence for such mechanisms were also recently reported by Takahashi and Walker (26).

To further characterize the role of XDrf1 during replication we followed its binding to chromatin and compared it to other replication factors as well as XDbf4 (Fig. 6A). The binding of XDrf1 to the chroma-
XCDC7/DRF1 Required for DNA Replication

tin occurred after Cdc6 and Mcm4 but before Cdc45 binding. Maximum XDrf1 binding correlated with maximum XCdc7 binding and was followed by Mcm4 phosphorylation on the chromatin (seen as an upward mobility shift, Fig. 6A) and maximum Cdc45 binding, which are markers of pre-RC activation (2, 5, 18). A small amount of XCdc7 and XDbf4 appeared to bind earlier than XDrf1. The level of XDbf4 on chromatin remained low until it increased significantly near the time of pre-RC activation (at 50 min). Addition of either an inhibitor of nuclear transport, wheat germ agglutinin, or a CDK inhibitor (roscovitine) to extracts did not affect XDrf1 and the majority of XCdc7 binding but blocked the second wave of XDbf4 binding to chromatin (Fig. 6A). However, XDrf1 association to the chromatin was completely blocked by the addition of geminin (a known pre-RCs assembly inhibitor) to the extract (27). The pre-RC dependent loading of XDrf1 onto chromatin was also established by Yanov et al. (9). As previously reported, we found the majority of XCdc7 binding to be pre-RC dependent but a small amount of XCdc7 remained bound to chromatin in the presence of geminin (3, 4). This small amount of XCdc7 binding is equivalent to the amount of XCdc7 that binds early and concurrently with a low level of XDbf4 on sperm chromatin. Accordingly, we confirmed that the early binding of XDbf4 to chromatin was also pre-RC independent and was not affected by geminin (13, 14) (Fig. 6A). Altogether these results suggest that both XCdc7 complexes bind to chromatin during replication. The binding of both complexes does not require nuclear transport or CDK activity and most likely occurs before the formation of a nuclear membrane around the sperm chromatin. First, a small amount of XCdc7/Dbf4 associates with the chromatin independently of pre-RCs assembly. It is followed by the recruitment of XCdc7/Drf1 that is entirely pre-RC dependent. The fact that most of the XCdc7 molecules appears to share the binding characteristics of XDrf1 indicates that there is an excess of XCdc7/Drf1 complexes over XCdc7/Dbf4 on the chromatin during replication. The timing of the additional XDbf4 binding and its sensitivity to CDK inhibitors suggests that it requires pre-RC activation.

In an effort to further understand how the depletion of XCdc7/Drf1 but not XCdc7/Dbf4 affects DNA replication in egg extracts, we compared the chromatin binding of several initiation factors in extracts depleted with anti-XCdc7, -XDrf1, -XDbf4, or non-immune antibodies (Fig. 6B). Depletion of the XCdc7/Dbf4 complex did not prevent the binding of XMcm4, XCdc7/Drf1 complex, or XCdc45 to the chromatin in agreement with our finding that this complex is not required for DNA replication. In contrast, XCdc7/Drf1 depletion had no effect on Mcm4 binding but dramatically decreased Cdc45 binding and Mcm4 phosphorylation. This effect reflects an inhibition of pre-RC activation and explains why XCdc7/Drf1 complexes are indispensable for the efficient replication of sperm chromatin in Xenopus egg extracts. The trace amounts of XCdc45 associated with chromatin in XDrf1-depleted extracts further supports the idea that XCdc7/Dbf4 binding to chromatin in the absence XCdc7/Drf1 is able to activate a few origins of replication. Our data also indicate that the binding of the two complexes to chromatin is independent of one another. Finally, as expected, simultaneous depletion of both complexes, by using anti-Cdc7 antibodies, completely blocked Cdc45 binding and as a result inhibited replication (Figs. 5B and 6B).

Chromatin Binding of XCdc7 Complexes Is Not Controlled by the ATR Checkpoint in Xenopus Egg Extracts—Several studies suggest that DDKs could be the target of DNA replication checkpoints (reviewed in Ref. 6). In Xenopus, the topoisomerase II inhibitor etoposide induces an ATR-dependent checkpoint that inactivates XCdc7/Dbf4 and prevents Cdc45 binding to the chromatin (15). In addition, XCdc7/Drf1 was shown to accumulate in an ATR-dependent manner during an aphidicolin-induced replication block and to inhibit Cdc45 binding to the chromatin (9). These two observations seem to be at odds with our results showing the predominant role of XCdc7/Drf1 and not XCdc7/Dbf4 in the binding of Cdc45 to chromatin in Xenopus egg extracts. Therefore, we examined the effects of aphidicolin and etoposide on the association of both complexes and Cdc45 to sperm chromatin in egg extracts (Fig. 7). As previously described, we found that the addition of 30 μM etoposide or 290 μM aphidicolin to egg extracts resulted in more than 90% inhibition of DNA replication (data not shown) and the ATR-dependent phosphorylation of the Chk1 protein (9, 15, 26) (Fig. 7). The loading of Cdc45 on the chromatin was slightly decreased in the presence of aphidicolin. Although etoposide did not seem to decrease Cdc45 binding in this 90-min chromatin assay, we found that in general etoposide also slightly decreased the maximum amount of Cdc45 on the chromatin during replication. This effect was better seen during binding kinetics as the peak of Cdc45 binding did not always occur at the same time in the control and in the presence of inhibitor (data not shown). We also confirmed that inhibition of ATR by caffeine in extracts containing etoposide or aphidicolin prevented Chk1 phosphorylation and stimulated Cdc45 binding to chromatin (9, 15, 28). However, the triggering of the ATR checkpoint in the presence of aphidicolin or etoposide had no significant effect on Mcm4 phosphorylation or the binding of XCdc7, XDrf1, and XDbf4 to chromatin; binding was also not affected by the addition of caffeine to aphidicolin- or etoposide-treated extracts. Despite the small inhibition of Cdc45 binding to chromatin, both inhibitors did not prevent Mcm4 phosphorylation or the activation of pre-RCs assembled on chromatin. Aphidicolin is known to allow the unwinding of replication origins assembled early on the chromatin but inhibits their elongation (29). Accordingly, the replication-dependent destruction of Cdt1 on the chromatin was completely inhibited by aphidicolin and the resulting blockage of the replication fork progression (30) (Fig. 7). Etoposide on the other hand, slowed down the destruction of Cdt1 but did not abolish it, indicating that a small amount of elongation occurred even though over 90% of DNA replication was inhibited. The uncoupling of unwinding and elongation at the origin in the presence of aphidicolin has been shown to generate the accumulation of single-stranded DNA coated with RPA that then induces the ATR checkpoint pathway (reviewed in Ref. 31). Our data suggest that etoposide triggered the ATR pathway in a similar fashion by slowing
down the progression of the replication fork. In summary, we found that the binding of the \textit{XCdc7}/\textit{Drf1} or \textit{XCdc7}/\textit{Dbf4} complexes to the chromatin does not seem to be regulated by ATR dependent pathways activated by partial or complete block of the replication fork.

**DISCUSSION**

In this study we have identified and characterized two regulatory subunits of the \textit{Xenopus} \textit{Cdc7} kinase; the \textit{XDbf4} and \textit{XDrf1} proteins. Whereas these subunits were not identified when we began this work, their existence has now been reported by several groups (9, 13, 14). The \textit{XDbf4} protein described in our study is identical to the one identified by Furukohri et al. (13) and Jares et al. (14). The \textit{XDrf1} clone we have identified encodes a protein that contains a longer N-terminal than the protein described by Yanow et al. (9). Based on sequence alignments between Drf1 proteins from different species, we believe that our protein is the \textit{bona fide} full-length \textit{XDrf1} protein. Our data indicate that the expression of \textit{XDrf1} and \textit{XDbf4} is differentially regulated during development and that contrary to previous reports \textit{XDrf1} and not \textit{XDbf4} is required for initiation of DNA replication in \textit{Xenopus} egg extracts (9, 13, 14). While this article was under revision similar observations were reported by Takahashi and Walter (26).

The restricted expression of \textit{XDrf1} to oogenesis and early embryos is characteristic of a maternal gene. Both \textit{XDrf1} mRNA and protein disappear after MTB at a time when the cell cycle is remodelled and gap phases appear between S and M phases (32, 33). This transition, which also corresponds to the activation of zygotic transcription, is often accompanied by deadenylation of maternal messages (34–36). Deadenylation is known to trigger translational silencing of numerous \textit{Xenopus} messages and also to induce their degradation in blastula stage embryos (37, 38). The \textit{XDrf1} 3’ untranslated region contains several sequences known to regulate the adenylation state of \textit{Xenopus} mRNAs (cytoplasmic polyadenylation element and AU-rich element), suggesting that the expression of \textit{XDrf1} is controlled by 3’ untranslated region-dependent mechanisms. Other initiation factors such as \textit{Xenopus} Mcm6, Mcm3, and \textit{Cdc7} are encoded by maternal genes and their expression follows a similar pattern to that of \textit{XDrf1} (25, 39). However, these maternal genes also have corresponding zygotic genes that encode distinct forms of the same proteins. The switch from the maternal to the zygotic form occurs after MBT when there is extensive cell cycle remodeling. Whereas our data provide no evidence supporting the existence of a zygotic \textit{XDrf1} gene, it is possible that our probes were unable to detect it. Indeed, our probes for Northern and Western analysis were directed against the C-terminal half of the coding region that is the least conserved region of the protein between species (8% identity between \textit{Xenopus} and \textit{Human Drf1}). The identification of \textit{Drf1} in various human and mouse tissues, as well as cell lines, supports the idea that a zygotic form of \textit{XDrf1} exists in late \textit{Xenopus} embryos and somatic cells (8, 10). Contrary to \textit{XDrf1}, our data suggest that there is only one \textit{XDbf4} gene that is expressed throughout development. Whereas the amount of \textit{XDbf4} message appears constant in embryos, the protein level increased after MBT suggesting that post-transcriptional mechanisms are likely to regulate \textit{XDbf4} protein expression.

The entire pool of \textit{XDrf1} and \textit{XDbf4} in egg extracts is engaged in individual complexes with \textit{XCdc7}. The concentration of each complex is equal to the concentration of their regulatory subunits. As a result, there is a 5-fold molar excess of \textit{XCdc7}/\textit{Drf1} over \textit{XCdc7}/\textit{Dbf4} in extracts. After establishing the presence of two \textit{Cdc7} complexes in egg extracts, our objective was to determine which complex is involved in the initiation of DNA replication. We demonstrated that \textit{XcCdc7}/\textit{Drf1} is the critical complex supporting pre-RC activation. Accordingly, the depletion of \textit{XcCdc7}/\textit{Dbf4} in egg extracts or in early embryos did not affect DNA replication (this study) or the division of embryonic cells before gastrulation (40). Although \textit{XcCdc7}/\textit{Dbf4} is not required for DNA replication in normal extracts, this complex can support a very small amount of Cdc45 binding in the absence of \textit{XcCdc7}/\textit{Drf1}. Considering that \textit{XcCdc7}/\textit{Dbf4} is an active kinase complex with a specific activity comparable with \textit{XcCdc7}/\textit{Drf1}, we believe that the inefficiency of \textit{XcCdc7}/\textit{Dbf4} to support DNA replication in extracts is linked to its low concentration and its inability to compete for pre-RC binding and activation. This conclusion agrees with our finding and the one by Jares et al. (14) that an excess of recombinant \textit{XcCdc7}/\textit{Dbf4} can restore DNA replication to a Cdc7-depleted extract. Even though \textit{XcCdc7}/\textit{Dbf4} is not necessary for replication in egg extracts, this still binds to chromatin in a two-stage process. First, \textit{XcCdc7}/\textit{Dbf4} binds early and before pre-RC assembly on the chromatin. Previous results also suggested that this binding is independent of origin recognition complex (13, 14). The second wave of \textit{XcCdc7}/\textit{Dbf4} binding requires pre-RC activation but not elongation as it is inhibited by CDK inhibitors but not by aphidicolin. Our observations differ significantly with the results of three different studies concluding that \textit{XDbf4} but not \textit{XDrf1} is required for DNA replication in \textit{Xenopus} egg extracts (9, 13, 14). We believe that the strength of our data resides in the experimental comparison of the two \textit{Cdc7} complexes and their specific and quantitative depletion from extracts. Whereas previous studies examined only one regulatory subunit at a time, they also reported depletions that seemed to be partial, leading to the dissociation of complexes or resulting in the inactivation of the extract (9, 13, 14).

Finally, we find that ATR-dependent checkpoints induced by aphidicolin or etoposide did not regulate the association of the two \textit{XcCdc7} complexes with the chromatin. Blocking elongation of replication by aphidicolin, in the presence or absence of ATR inhibitor, did not affect the amount of either complex on the chromatin. Our data do not support a previous model in which ATR-dependent accumulation of \textit{XDrf1} on the chromatin inhibits \textit{XcCdc45} binding (9). To the contrary, we show that \textit{XDrf1} is required for \textit{XcCdc45} chromatin association. In our experiments, the ATR checkpoint induced by etoposide resulted from a partial block of elongation. The slowing down of the fork progression by etoposide has also been observed by Lucas et al. (41). Unlike Constanzzo et al. (15), we were not able to activate an ATR checkpoint independently of pre-RC activation, leading to the dissociation of the \textit{XcCdc7}/\textit{Dbf4} complex and the prevention of \textit{XDbf4} and \textit{XcCdc45} binding to chromatin. The reasons for this discrepancy are unknown as similar experimental conditions were used in both studies. Nevertheless, it is unclear how an ATR-dependent dissociation of \textit{XcCdc7}/\textit{Dbf4} would affect the loading of \textit{XcCdc45} that only requires the \textit{XcCdc7}/\textit{Drf1} complex.

The presence of two \textit{Xenopus} \textit{Cdc7} complexes during early development while only one is required for embryonic cell division is puzzling. \textit{XcCdc7}/\textit{Drf1} supports DNA replication at least until gastrulation but the role of \textit{XcCdc7}/\textit{Dbf4}, if any, during that time is unknown. The accumulation of \textit{XDbf4} during maturation could indicate that the \textit{XcCdc7}/\textit{Dbf4} complex is required for meiosis. Alternatively, the potential involvement of \textit{XcCdc7}/\textit{Dbf4} in sister chromatid cohesion, origin recognition complex localization, or gene regulation, as shown in different organisms, could explain why the complex binds to chromatin in a pre-RC independent manner (12, 40, 42, 43). Additional studies will be needed to define the role of \textit{XcCdc7}/\textit{Dbf4} during the early embryonic cell cycle and determine whether a zygotic version of the \textit{XcCdc7}/\textit{Drf1} complex is also required for initiation of DNA replication during the somatic cell cycle.
### REFERENCES

1. Takeda, D. Y., and Dutta, A. (2005) *Oncogene* **24**, 2827–2843
2. Mimura, S., and Takisawa, H. (1998) *EMBO J.* **17**, 5699–5707
3. Jares, P., and Blow, J. J. (2000) *Genes Dev.* **14**, 1528–1540
4. Walter, J. C. (2000) *J. Biol. Chem.* **275**, 39773–39778
5. Pacek, M., and Walter, J. C. (2004) *EMBO J.* **23**, 3667–3676
6. Kim, J. M., Yamada, M., and Masai, H. (1998) *EMBO J.* **17**, 5699–5707
7. Nakamura, T., Kishida, M., and Shimoda, C. (2000) *Genes Cells* **5**, 463–479
8. Montagnoli, A., Bosotti, R., Villa, F., Rialland, M., Berthelsen, J., and Santocanale, C. (2002) *EMBO J.* **21**, 3171–3181
9. Yanow, S. K., Gold, D. A., Yoo, H. Y., and Dunphy, W. G. (2003) *J. Biol. Chem.* **278**, 41083–41092
10. Yoshizawa-Sugata, N., Ishii, A., Taniyama, C., Matsui, E., Arai, K., and Masai, H. (2005) *J. Biol. Chem.* **280**, 13062–13070
11. Nakamura, T., Nakamura-Kubo, M., and Shimoda, C. (2002) *Mol. Cell. Biol.* **22**, 309–320
12. Hodson, J. A., Bailis, J. M., and Forsburg, S. L. (2003) *Nucleic Acids Res.* **31**, e134
13. Furukohri, A., Sato, N., Masai, H., Arai, K., Sugino, A., and Waga, S. (2003) *J. Biochem.* (Tokyo) **134**, 447–457
14. Jares, P., Luciani, M. G., and Blow, J. J. (2004) *BMC Mol. Biol.* **5**, 5
15. Costanzo, V., Shechter, D., Lupardus, P. J., Cimprich, K. A., Gottesman, M., and Gautier, J. (2003) *Mol. Cell* **11**, 203–213
16. Dumont, J. N. (1972) *J. Morphol.* **136**, 153–179
17. Nieuwkoop, P. D. (1977) *Curr. Top. Dev. Biol.* **11**, 115–132
18. Pereverzeva, I., Whitmire, E., Khan, B., and Coue, M. (2000) *Mol. Cell. Biol.* **20**, 3667–3676
19. Whitmire, E., Khan, B., and Coue, M. (2002) *Nature* **419**, 722–725
20. Coue, M., Kearsey, S. E., and Mechali, M. (1996) *EMBO J.* **15**, 1085–1097
21. Murray, A. W. (1991) *Methods Cell Biol.* **36**, 581–605
22. Lohka, M. J., and Masui, Y. (1983) *Science* **220**, 719–721
23. Menut, S., Lemaître, J. M., Hair, A., and Mechali, M. (1999) *Advances in Molecular Biology*, pp. 196–226, Oxford University Press, Oxford
24. Masai, H., and Arai, K. (2000) *Biochem. Biophys. Res. Commun.* **275**, 228–232
25. Roberts, B., Ying, C. Y., Gautier, J., and Maller, J. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2808–2814
26. Takahashi, T. S., and Walter, J. C. (2005) *Genes Dev.* **19**, 2295–2300
27. McGarry, T. J., and Kirschner, M. W. (1998) *Cell* **93**, 1043–1053
28. Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C., and Cimprich, K. A. (2005) *Genes Dev.* **19**, 1040–1052
29. Walter, J., and Newport, J. (2000) *Mol. Cell* **5**, 617–627
30. Arias, E. E., and Walter, J. C. (2005) *Genes Dev.* **19**, 114–126
31. Cortez, D. (2005) *Genes Dev.* **19**, 1007–1012
32. Newport, J., and Kirschner, M. (1982) *Cell* **30**, 687–696
33. Newport, J., and Dasso, M. (1989) *J. Cell Sci. Suppl.* **12**, 149–160
34. Audic, Y., Anderson, C., Bhatti, R., and Hartley, R. S. (2001) *Mol. Cell. Biol.* **21**, 1662–1671
35. Paris, J., and Philippe, M. (1990) *Dev. Biol.* **140**, 221–224
36. Legagneux, V., Omilli, F., and Osborne, H. B. (1995) *RNA* (N.Y.) **1**, 1001–1008
37. Audic, Y., Omilli, F., and Osborne, H. B. (1997) *Mol. Cell. Biol.* **17**, 209–218
38. Voeltz, G. K., and Steitz, J. A. (1998) *Mol. Cell. Biol.* **18**, 7537–7545
39. Sible, J. C., Erickson, E., Hendrickson, M., Maller, J. L., and Gautier, J. (1998) *Curr. Biol.* **8**, 347–350
40. Brott, B. K., and Sokol, S. Y. (2005) *Dev. Cell* **8**, 703–715
41. Lucas, I., Germe, T., Chevrier-Miller, M., and Hyrien, O. (2001) *EMBO J.* **20**, 6509–6519
42. Takeda, T., Ogino, K., Tatebayashi, K., Ikeda, H., Arai, K., and Masai, H. (2001) *Mol. Biol. Cell* **12**, 1257–1274
43. Zhang, H., and Tower, J. (2004) *Development* **131**, 2089–2099