

Cyclin E Uses Cdc6 as a Chromatin-associated Receptor Required for DNA Replication[Ⓢ]

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Abstract. Using an in vitro chromatin assembly assay in *Xenopus* egg extract, we show that cyclin E binds specifically and saturably to chromatin in three phases. In the first phase, the origin recognition complex and Cdc6 pre-replication proteins, but not the minichromosome maintenance complex, are necessary and biochemically sufficient for ATP-dependent binding of cyclin E–Cdk2 to DNA. We find that cyclin E binds the NH₂-terminal region of Cdc6 containing Cy–Arg–X–Leu (RXL) motifs. Cyclin E proteins with mutated substrate selection (Met–Arg–Ala–Ile–Leu; MRAIL) motifs fail to bind Cdc6, fail to compete with endogenous cyclin E–Cdk2 for chromatin binding, and fail to rescue replication in cyclin E–depleted extracts. Cdc6 proteins with mutations in the three consensus RXL motifs are quantitatively deficient for cyclin E binding and for rescuing replication in Cdc6-depleted extracts. Thus, the cyclin E–Cdc6 interaction that localizes the Cdk2 complex to chromatin is important for DNA replication.

During the second phase, cyclin E–Cdk2 accumulates on chromatin, dependent on polymerase activity. In the third phase, cyclin E is phosphorylated, and the cyclin E–Cdk2 complex is displaced from chromatin in mitosis. In vitro, mitogen-activated protein kinase and especially cyclin B–Cdc2, but not the polo-like kinase 1, remove cyclin E–Cdk2 from chromatin. Rebinding of hyperphosphorylated cyclin E–Cdk2 to interphase chromatin requires dephosphorylation, and the Cdk kinase–directed Cdc14 phosphatase is sufficient for this dephosphorylation in vitro. These three phases of cyclin E association with chromatin may facilitate the diverse activities of cyclin E–Cdk2 in initiating replication, blocking rereplication, and allowing resetting of origins after mitosis.

Key words: cyclin-dependent kinases • origin recognition complex • DNA replication • Cdc6 • Cdc14

Introduction

The requirements for determining the timing and origin selection for eukaryotic DNA replication are now being intensively investigated. In yeast, origin selection requires the origin recognition complex (ORC)¹ to bind initiation sites on DNA (Bell and Stillman, 1992; Rao and Stillman, 1995). Although such initiation sequences are not well defined in higher eukaryotes, it is likely that ORC homologues serve a similar function in these organisms (Carpenter et al., 1996). Studies in *Xenopus* egg extracts and mammalian cells show that ORC recruits Cdc6 and the minichromosome maintenance (MCM) complex to chromatin (Coleman et al., 1996) and that these preinitiation factors are essential for gener-

ating functional origins (Yan et al., 1991; Liang et al., 1995; Romanowski et al., 1996; Williams et al., 1997). The MCM proteins have also been implicated in limiting DNA replication to a single round per cell cycle (Tye, 1994; Chong et al., 1995; Kubota et al., 1995). It is thought that the MCM complex is stripped from chromatin as DNA polymerase moves with the replication fork, thereby removing replication competence from origins that have fired.

The cyclin E–Cdk2 complex is essential for timing initiation of DNA replication (Knoblich et al., 1994; Strausfeld et al., 1994; Jackson et al., 1995) and has been implicated in rereplication control, as high levels of cyclin E appear to block the licensing of origins in *Drosophila* and *Xenopus* (Hua et al., 1997; Follette et al., 1998; Weiss et al., 1998). Concomitant with the initiation of DNA replication, cyclin E is concentrated ~200-fold within the nucleus after nuclear assembly (Chevalier et al., 1996; Hua et al., 1997). The concentration of essential factors such as cyclin E is a central function of the nucleus in DNA replication (Walter et al., 1998; Swanson et al., 2000).

How cyclin E–Cdk2 promotes DNA replication remains unclear, because we do not know its relevant substrates,

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¹Abbreviations used in this paper: CIP, calf intestinal phosphatase; GST, glutathione S-transferase; HSS, high speed supernatant; IP, immunoprecipitation; IVT, in vitro translated; LSS, low speed supernatant; MAP, mitogen-activated protein; MCM, minichromosome maintenance; MOI, multiplicity of infection; MRAIL, Met–Arg–Ala–Ile–Leu; ORC, origin recognition complex; Plk1, polo-like kinase 1; RXL, Arg–X–Leu.

how those substrates are selected, or how phosphorylation by cyclin E–Cdk2 changes their ability to promote replication. Candidates for cyclin E–Cdk2 substrates have been described, including the protein NPAT (Zhao et al., 1998). Studies from fission yeast show that the Cdc6 homologue, Cdc18, is phosphorylated by a cyclin-dependent kinase at the G1/S transition (Jallepalli et al., 1997) and, indeed, human and *Xenopus* Cdc6 are good *in vitro* substrates for Cdk2 kinases (Jiang et al., 1999; Petersen et al., 1999). Phosphorylation of Cdc6 by a Cdk2 complex in human cells appears to relocalize the Cdc6 protein from the nucleus to the cytoplasm (Saha et al., 1998; Jiang et al., 1999; Petersen et al., 1999). Although this relocalization is speculated to inactivate Cdc6 after replication initiation, the specific connection to replication remains unproven.

The ability of cyclin–Cdk complexes to select their specific substrates is determined in part by binding of the cyclin to regions on the substrate. The crystal structure of human cyclin A–Cdk2 bound to the inhibitor/substrate p27^{Kip1} defined a region of the cyclin A protein that interacts directly with p27 (Russo et al., 1996). This region contains the Met-Arg-Ala-Ile-Leu (MRAIL) motif conserved among cyclin A and cyclin E homologues in many organisms and forms a hydrophobic binding pocket that interacts with an Arg-X-Leu (RXL) peptide within p27. The RXL motif itself is conserved among many cyclin E and cyclin A substrates, including p21, E2F, and p107 (Adams et al., 1996; Chen et al., 1996), suggesting that the RXL motifs are a signature for cyclin–Cdk2 targets. RXL motifs are often surrounded by consensus CDK phosphorylation sites, as is the case for Cdc6 (Jiang et al., 1999; Petersen et al., 1999).

We were interested in further understanding the mechanisms governing cyclin E–Cdk2 control of DNA replication. Because cyclin E–Cdk2 likely phosphorylates chromatin-associated prereplication proteins, we speculated that cyclin E might function on chromatin. Here, we show that cyclin E–Cdk2 associates with chromatin in three phases and that this association in the first phase depends primarily on the prior recruitment of the ORC–Cdc6 complex. We further show that the cyclin E–Cdk2–Cdc6 interaction is a direct association mediated by the MRAIL motif in cyclin E and the RXL motif, and possibly another site in the NH₂ terminus of Cdc6, and that this interaction is essential for the initiation of DNA replication. In the second phase, cyclin E–Cdk2 accumulates on chromatin as replication proceeds, potentially explaining the ability of cyclin E–Cdk2 to block rereplication. We find this accumulation requires polymerase activity. In the third phase, the cyclin E–chromatin interaction is abolished in mitosis and reestablished upon the exit from mitosis, thereby allowing a new round of replication. We have found that cyclin B–Cdc2 and, to some extent, mitogen-activated protein (MAP) kinase are capable of phosphorylating cyclin E in mitosis and removing it from chromatin, and that Cdc14, a phosphatase essential for the exit from mitosis, is capable of reversing the mitotic phosphorylation of cyclin E and allowing it to rebind chromatin in G1. Thus, the cell cycle-regulated three-phase association of cyclin E with its chromatin receptor may help explain the coordination of its functions in initiating replication, blocking rereplication, and relicensing origins.

Materials and Methods

Preparation of *Xenopus* Egg Extracts and Sperm Nuclei

For interphase extracts, dejellied eggs were rinsed in ELB (250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Hepes, pH 7.7, 1 mM DTT, 50 μg/ml cycloheximide, 10 μg/ml cytochalasin D), and centrifuged (13,000 rpm, 10 min). Cytosol was recentrifuged (24,000 rpm, 10 min), and the supernatant was removed with a syringe and kept on ice; the second spin significantly improved replication efficiency. Cycling extracts were made similarly, except that eggs were activated by the calcium ionophore A23187 (Sigma-Aldrich), and cycloheximide was omitted from the buffer (Murray and Kirschner, 1989). For chromatin assembly assays, high speed supernatants (HSSs) were made similarly, except that XB (50 mM sucrose, 100 mM KCl, 100 μM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, pH 7.7) was substituted for ELB, an energy regenerating system was added, and centrifugation (100,000 g, 30 min) was performed to remove membranes (Murray et al., 1989). Sperm nuclei were isolated as described (Jackson et al., 1995).

Sedimentation Assays to Isolate Assembled Chromatin from HSS and Low Speed Supernatants

HSS Reactions. HSS for chromatin assembly was made as described above. Reactions were carried out by incubating 20 μl of interphase HSS with 20 ng of sperm DNA or 1 μg of λ DNA, diluted to 50 μl with XB2 (XB with 2 mM MgCl₂). In some experiments, baculovirus-expressed cyclin E–Cdk2 was added to 200 nM. Inhibitors or recombinant proteins were preincubated with HSS for 15 min before DNA template addition. Upon DNA addition, reactions were incubated (30 min, 22°C), stopped by dilution (150 μl of cold XB2), layered on a 400-μl cushion (1.1 M sucrose in XB2), and spun (11,000 rpm, 30 min, 4°C) in a SW50.1. The gradient interface was washed with XB2 to remove unpelleted material, and sample buffer was added to the pellet for SDS-PAGE.

LSS Reactions. Low speed supernatant (LSS) was supplemented with an energy regenerating system before sperm addition (1,000 sperm/μl). Samples were incubated (23°C) for the indicated times, diluted with five volumes of cold ELB, layered over a 0.5-M sucrose cushion, and centrifuged in a Beckman 152 microfuge (20 s). Pelleted nuclei were resuspended in sample buffer and analyzed by Western blotting. Chromatin was extracted from a duplicate set of assembled nuclei by adding 10 volumes of chromatin extraction buffer (50 mM KCl, 50 mM Hepes, pH 7.7, 5 mM MgCl₂, 5 mM EGTA, 2 mM β-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine, 0.1% NP-40), mixing gently, and leaving on ice for 30 min, before respinning the tubes as above. Similar assays show the association of replication proteins with chromatin templates (Chong et al., 1995; Yan and Newport, 1995; Martinez-Campa et al., 1997).

Samples treated with mitotic kinases were assembled in LSS (1 h) and murine MAP kinase, human cyclin B–Cdc2 (1 U each; New England Biolabs, Inc.), or glutathione S-transferase (GST)–XPlk1 (a gift from Jan-Michael Peters, Institute of Molecular Pathology, Vienna, Austria) were added for 10 min. Chromatin fractions were isolated as above.

Replication Assays

10 μl of cycloheximide-stabilized interphase extract was mixed with 3–5 ng of sperm, and replication assays were performed and quantitated as described (Jackson et al., 1995).

Phosphorylation/Dephosphorylation Reactions

2.5 μg of bacterially expressed, purified GST–Xcyclin E was incubated with 1 U of MAP kinase, cyclin B–Cdc2, GST–Plk1, or baculovirus-expressed cyclin E–Cdk2 in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 100 μM ATP) in the presence of 0.15 μCi of γ[³²P]ATP. After 30 min at 30°C, half of each sample was removed and supplemented with 2 μM GST–Cdc14. Cdc14-treated and -untreated samples were incubated (30 min, 30°C) before stopping reactions with sample buffer, resolving by SDS-PAGE, and visualizing phosphorylated GST–cyclin E by autoradiography.

Calculation of the Number of Cyclin E Molecules Per Origin

The concentration of cytosolic cyclin E–Cdk2 required for binding to chromatin was estimated by adding baculovirus-expressed cyclin E–Cdk2 to DNA in cyclin E-depleted HSS. The number of molecules per origin represented by this binding event was calculated by determining the per-

centage of cyclin E that bound to DNA by quantitative Western blotting. Assumptions include that the number of origins per nucleus equals 10^5 (Walter and Newport, 1997), the volume of a nucleus equals 2.5 nl (Hua et al., 1997), and the concentration of cyclin E in cytosolic extract equals 60 nM and in nuclei equals 12 μ M (Jackson et al., 1995; Hua et al., 1997). Thus, $60 \text{ nmol/liter cyclin E-Cdk2} \times (6 \times 10^{23} \text{ molecules}) \times (2.5 \times 10^{-9} \text{ l/liter nuclei}) = (9 \times 10^7 \text{ molecules/nuclei})$. Because $\sim 0.1\%$ of the cyclin E from HSS binds to chromatin, we estimate that $\sim 1 \times 10^5 \text{ molecules/nuclei per } 10^5 \text{ origins/nucleus} = \sim 1 \text{ molecule cyclin E/origin}$.

To determine the maximum capacity of chromatin for cyclin E, known amounts of baculovirus cyclin E-Cdk2 were titrated into cyclin E-depleted LSS extracts and chromatin-associated cyclin E, measured by quantitative Western blot. The maximal level was roughly equal to the amount of endogenous cyclin E bound to chromatin immediately before mitosis.

In Vitro Binding Assays

GST fusion proteins of either human p21N, human p21C, human p27, XCdc6N, XCdc6C, or human Cdc14, added to a concentration of 1 μ M, were mixed with baculovirus-expressed Xcyclin E-XCdk2 (0.4 μ M) and diluted to 10 μ l with XB⁻. Mixtures were incubated (1 h, 25°C), diluted with 90 μ l of immunoprecipitation (IP) buffer (100 mM NaCl, 50 mM β -glycerophosphate, 5 mM EDTA, 0.1% Triton X-100, pH 7.2), and spun (13,000 g, 10 min). Supernatants were added to glutathione-agarose and rocked (30 min, 4°C). Beads were washed with IP buffer, resuspended in sample buffer, and resolved by SDS-PAGE.

Mutants of Xcyclin E were created by PCR mutagenesis and verified by sequencing as M143A L147A W150A and L186A Q187A. RXL mutants of XCdc6 were engineered and verified as (a) R93A L94A L95A, (b) R165A L167A, and (c) R258A L260A.

In vitro-translated (IVT) ³⁵S-labeled cyclin E was expressed from pGEM3Zl⁺ using the TNT-coupled reticulocyte lysate systems (Promega).

Purification of the XORC Complex from Xenopus laevis Extract

ORC was purified 500-fold from HSS made from the eggs of 50 frogs (Rowles et al., 1996).

Immunodepletion and ATP Depletion

Immunodepletions were performed by binding crude (Xcyclin E) or affinity-purified (XCdc6) rabbit sera to protein A-Sepharose beads for 1 h. Antibody beads were incubated with extract ($2 \times 45 \text{ min}$, 4°C) and then centrifuged (13,000 rpm, 10 min). Control depletions were performed with beads alone. ATP depletion was performed by adding hexokinase beads (Sigma-Aldrich), and residual ATP was determined to be $<3\%$ by luciferase assay.

Antibody Production and Purification

Purified GST-XORC2, GST-XORC1, GST-Xcyclin E, and GST-XCdc6 were used to raise antisera in rabbits (Josman Immunoresearch). Affinity purification of antisera was performed by acid elution from MBP fusion proteins coupled to CNBr-activated Sepharose. Anti-Cdk2 antibodies have been previously described (Jackson et al., 1995).

Production of Bacterially Expressed GST and MBP Proteins and Baculovirus-expressed Cyclin E-Cdk2 and Cdc6

GST and MBP fusion proteins were expressed in BL21 pLysS and purified over glutathione or amylose resins as described (Jackson et al., 1995).

The following fragments were made as GST or MBP fusions: p21N, amino acids 1-90; p21C, amino acids 87-164 (Chen et al., 1995); and Cdc6N, amino acids 2-168; Cdc6C, amino acids 169-554 (provided by D. Wolf, Harvard School of Public Health, Boston, MA).

Production of baculovirus-expressed His-XCdc6 was performed by infecting Sf9 cells with the XCdc6 virus (a gift from Bill Dunphy, California Institute of Technology, Pasadena, CA) and purifying over Ni-NTA resin (QIAGEN).

Baculovirus-expressed Xcyclin E-His-XCdk2 (a gift of Jim Maller, University of Colorado, Denver, CO) was produced by coinfection with His-XCdk2 virus (multiplicity of infection [MOI] = 10) and Xcyclin E virus (MOI = 15) to favor cyclin E-Cdk2 complex formation (Strausfeld et al., 1996). Autophosphorylated cyclin E was produced by coinfection with Cdk2 at a high MOI for both viruses in the presence of high concentrations of ATP ($\sim 1 \text{ mM}$).

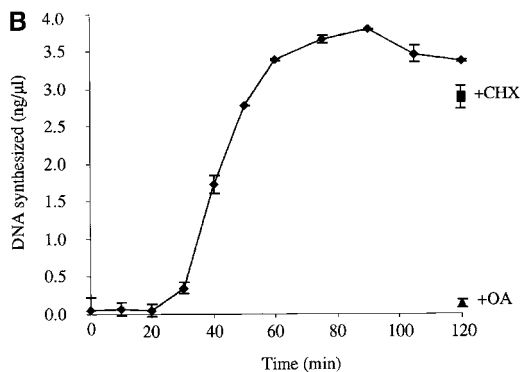
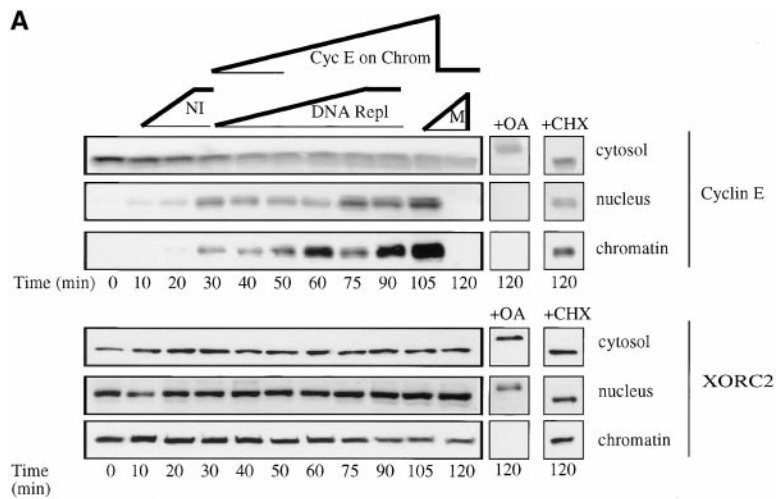


Figure 1. Cyclin E associates with chromatin in LSS after nuclear import. (A) Sperm chromatin was assembled in the presence of cycling LSS at 23°C for 0–2 h (time of assembly shown beneath blots) before spinning through a sucrose cushion to isolate nuclei in duplicate. One nuclear sample was extracted with chromatin extraction buffer and respun to isolate chromatin-associated proteins. Cytosolic, nuclear, and chromatin-associated samples were resolved by SDS-PAGE and analyzed by Western blotting with ORC or cyclin E antibodies. Schematics above blots depict the timing of relevant events including, nuclear import (NI), DNA replication (DNA repl), cyclin E association with chromatin (Cyc E on Chrom), and mitosis (M). The indicated samples were supplemented with 10 μ M okadaic acid (OA) or 100 μ g/ml cycloheximide (CHX) for 120 min. (B) Samples identical to those in A were supplemented with [α -³²P]dCTP. At each time point, the reactions were stopped, and the amount of DNA synthesized in duplicate samples was quantitated as detailed in Materials and Methods.

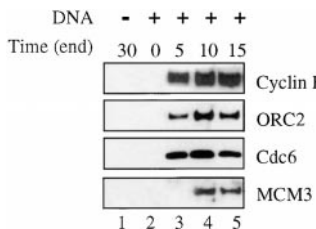


Figure 2. The cyclin E-Cdk2 complex from HSS associates with chromatin with kinetics similar to ORC and Cdc6, but earlier than MCM3. Chromatin was assembled by addition of sperm DNA to HSSs from *Xenopus* egg extracts, and reactions were

stopped at indicated times. Chromatin templates were isolated, resolved by SDS-PAGE, and analyzed by Western blotting with antibodies to *Xenopus* ORC2, Cdc6, MCM3, and cyclin E (Materials and Methods). Lane 1, no DNA, 30 minutes; lanes 2–5, DNA templates assembled for 0, 5, 10, or 15 min. Later time points showed no additional assembly of ORC, Cdc6, MCM3, or cyclin E-Cdk2.

Western Blotting

Western blotting was performed as described (Jackson et al., 1995). Affinity-purified antibodies were used at 0.5–1.0 $\mu\text{g/ml}$; crude sera was used as indicated: ORC2 antisera (1:2,500), ORC1 antisera (1:2,000). Crude MCM3 antisera (1:3,000) was a gift from Ron Laskey (Romanowski et al., 1996).

Online Supplemental Material

Deletion mutant analysis was used to map an NH_2 -terminal region of XCdc6 outside of the RXL motif that interacts with Xcyclin E. Supplemental Figure S1 depicts experiments assessing the ability of XCdc6 NH_2 -terminal deletion mutants to bind to cyclin E, become phosphorylated by cyclin E-Cdk2, and sustain replication. Figure S1 is available at <http://www.jcb.org/cgi/content/full/152/6/1267/DC1>

Results

Cyclin E-Cdk2 Is Recruited to Chromatin after Nuclear Accumulation and Is Removed from Chromatin in Mitosis

To study the ordered events of DNA replication, we optimized an assay to isolate chromatin templates assembled within nuclei formed in LSS of *Xenopus* egg extracts. These cycling extracts recapitulate the events of the mitotic cell cycle in vitro. First, we separated sperm nuclei assembled in LSS from the cytosolic fraction by centrifugation (Fig. 1 A). We extracted purified nuclei with chromatin extraction buffer and recentrifuged to separate nucleoplasmic proteins from tightly chromatin-associated proteins. Similar assays have been performed in several systems to study the association of replication proteins with chromatin templates (Materials and Methods). The amount of DNA replication completed at each time point is shown for reference (Fig. 1 B). Because cyclin E-Cdk2 promotes DNA replication, we tested whether cyclin E-Cdk2 directly interacts with chromatin. We found that cyclin E-Cdk2 associated with chromatin assembled in cycling LSS extracts (Fig. 1 A). In this first phase, cyclin E-Cdk2 was imported into the nucleus after nuclear assembly and bound to chromatin immediately after nuclear import, unlike ORC and Cdc6, which associated with chromatin before nuclear formation (Fig. 1 A). Cyclin E became detergent-inextractable at the same time that MCMs appear in the detergent-extracted chromatin fractions (not shown).

In a second phase, cyclin E continued to accumulate on chromatin throughout replication (Fig. 1 A).

In a third phase, chromatin binding of cyclin E-Cdk2 was mitotically regulated. When cyclin B-Cdc2 kinase activity peaked (indicated by the triangle containing an M), cyclin E-Cdk2 was rapidly displaced from chromatin (Fig. 1 A). Although we saw displacement of XORC1 and XORC2 later in mitosis (not shown), XORC2 appeared to be more stably associated with chromatin in early mitosis (Fig. 1 A) when nuclear envelope breakdown was first initiated. Addition of the phosphatase 2A inhibitor okadaic acid to interphase extracts also induced the mitotic state (Lee et al., 1991) and displaced both cyclin E and XORC from chromatin. Inhibition of cyclin B synthesis and mitotic entry with the protein synthesis inhibitor cycloheximide blocked cyclin E-Cdk2 displacement. Because DNA replication does not require protein synthesis in LSSs, this indicates that the mitotic state, rather than completion of DNA replication, displaces cyclin E-Cdk2 from chromatin. Cyclin E also appears to be more sensitive to mitotic signals for chromatin displacement than XORC.

A Chromatin Assembly Assay Shows That Cyclin E Associates with Chromatin with Kinetics Similar to ORC and Cdc6

To study the first phase of cyclin E-Cdk2 binding to interphase chromatin, we optimized an assay to isolate *Xenopus* sperm or λ DNA templates assembled in HSSs of interphase egg extracts (Swedlow and Hirano, 1996). In these extracts, prereplication complexes form, but events after prereplication complex formation are blocked because the extract lacks membranes and cannot assemble nuclei. We find that *Xenopus* sperm and λ DNA behave identically in all of our HSS assays, which were each repeated using both templates to verify results. The DNA templates used are noted in the figure legends. After chromatin assembly, reactions were overlaid on a sucrose cushion and chromatin isolated by sedimentation. The chromatin-associated proteins were resolved by SDS-PAGE and examined by Western blotting. The assay was optimized to ensure a high efficiency of isolating the chromatin templates (>95%) and to minimize nonspecific sedimentation of cytoskeletal proteins (Materials and Methods).

In this assay, ORC and Cdc6 associated with chromatin within 5 min, whereas assembly of MCM proteins was consistently delayed, requiring ~ 10 min (Fig. 2). Using sperm or λ DNA, we found the kinetics of assembly were indistinguishable. Single-stranded M13 DNA or RNA was unable to bind preinitiation factors in this assay.

We found that the endogenous cyclin E-Cdk2 complex bound to chromatin with kinetics similar to ORC and Cdc6 (Fig. 2). On chromatin, cyclin E appeared as a doublet, although the fastest migrating, hypophosphorylated form (see Fig. 9 B), bound most readily. Quantitative Western blotting indicated that the level of cyclin E-Cdk2 binding to chromatin was approximately one molecule/origin (see Materials and Methods). This low level of cyclin E was difficult to detect and required exposing the blot shown in Fig. 2 overnight. Addition of exogenous cyclin E-Cdk2 purified from baculovirus increased the total amount of cyclin E-Cdk2 bound to chromatin (Fig. 3 B), suggesting that the number of cyclin E-Cdk2 chromatin receptors are in excess in HSS extracts. Nonetheless, addition of excess cy-

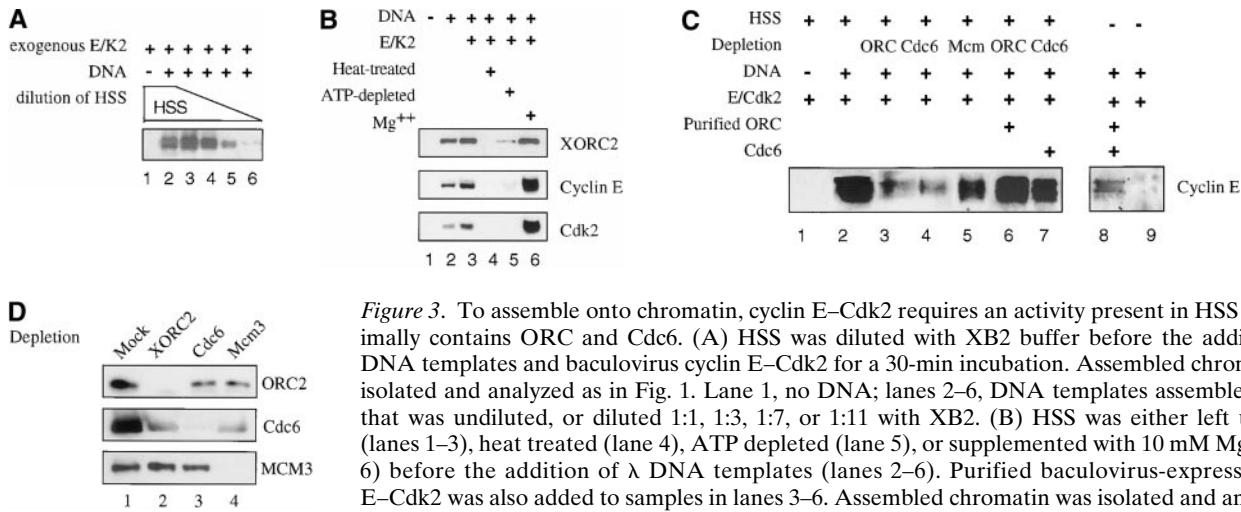


Figure 3. To assemble onto chromatin, cyclin E–Cdk2 requires an activity present in HSS that minimally contains ORC and Cdc6. (A) HSS was diluted with XB2 buffer before the addition of λ DNA templates and baculovirus cyclin E–Cdk2 for a 30-min incubation. Assembled chromatin was isolated and analyzed as in Fig. 1. Lane 1, no DNA; lanes 2–6, DNA templates assembled in HSS that was undiluted, or diluted 1:1, 1:3, 1:7, or 1:11 with XB2. (B) HSS was either left untreated (lanes 1–3), heat treated (lane 4), ATP depleted (lane 5), or supplemented with 10 mM MgCl₂ (lane 6) before the addition of λ DNA templates (lanes 2–6). Purified baculovirus-expressed cyclin E–Cdk2 was also added to samples in lanes 3–6. Assembled chromatin was isolated and analyzed as above. (C) Individual aliquots of HSS were immunodepleted with antibodies specific to XORC2

(lanes 3 and 6), XCdc6 (lanes 4 and 7), XMCM3 (lane 5), or with beads alone (lane 2). Specific samples were supplemented with purified XORC complex (lanes 6 and 8) or baculovirus-expressed XCdc6 (lanes 7 and 8). All samples included baculovirus-expressed Xcyclin E–Cdk2 and an energy regenerating system. Depleted samples with and without additions were incubated with λ DNA for 30 min, sedimented through a sucrose cushion, and resolved by SDS-PAGE. (D) Western blots of depleted HSS used for assembling chromatin in C. Lane 1, mock depleted; lane 2, ORC2 depleted; lane 3, Cdc6 depleted; lane 4, MCM3 depleted.

clin E–Cdk2 did not accelerate cyclin E assembly onto chromatin, suggesting that binding of cyclin E–Cdk2 to chromatin depends on the prior assembly of other factors.

Assembly of Cyclin E–Cdk2 onto Chromatin Requires an ATP-dependent Factor in HSS

To determine the requirements for the first phase of cyclin E–Cdk2 binding to chromatin, we incubated a fixed amount of purified baculovirus cyclin E–Cdk2 and λ DNA template with dilutions of HSS, and isolated the assembled chromatin templates. Cyclin E was unable to assemble onto the DNA template in the absence of HSS, but increasing the concentration of HSS caused a linear increase in the amount of cyclin E–Cdk2 assembled onto chromatin (Fig. 3 A), suggesting that extract contains an activity that promotes cyclin E binding to chromatin, which we term a “chromatin receptor.”

We determined the biochemical requirements for cyclin E–Cdk2 recruitment to DNA (Fig. 3 B). Heat treatment or ATP depletion of the extract caused a complete loss of cyclin E–Cdk2 binding to chromatin. ATP depletion (97%) also strongly reduced binding of ORC (Fig. 3 B) and Cdc6 (not shown), although a small amount of residual ORC binding to chromatin was observed, likely due to residual ATP-loaded ORC remaining after ATP depletion. Direct binding of yeast ORC to DNA requires ATP (Bell and Stillman, 1992). Addition of excess Mg²⁺ stimulated the assembly of cyclin E–Cdk2 onto chromatin but not ORC binding (Fig. 3 B). Finally, Cdk activity is not required for recruitment, because addition of the chemical Cdk inhibitor roscovitine had no effect on cyclin E chromatin recruitment (not shown). In contrast, protein Cdk inhibitors, including p21^{Cip1} or *Xenopus* p27^{Xic1} (1 μ M), did inhibit cyclin E recruitment to chromatin (not shown), likely indicating that they compete with the endogenous receptor protein(s) for binding to cyclin E (see below).

The ORC–Cdc6 Preinitiation Complex Acts as a Receptor for Cyclin E–Cdk2 on Chromatin

To determine whether preinitiation factors facilitated cyclin E–Cdk2 chromatin recruitment, we depleted ORC, Cdc6, or MCM proteins from HSS before the addition of purified cyclin E–Cdk2 and DNA. When the assembled chromatin templates were isolated from these samples, we found that a substantial fraction (~80%) of the cyclin E–Cdk2 binding was lost in the absence of ORC and Cdc6, whereas MCM depletion had no significant effect on binding (Fig. 3 C). After depletion of ORC or Cdc6, ~20% of cyclin E–Cdk2 did bind to chromatin, even though ORC and Cdc6 depletions appeared quantitative (Fig. 3 D, >95%). Therefore, we suspect that the ORC–Cdc6 complex may not be the only receptor for cyclin E–Cdk2 on chromatin (see Discussion). Purified *Xenopus* ORC and recombinant XCdc6 rescued cyclin E binding to chromatin from ORC- or Cdc6-depleted extracts (Fig. 3 C). Surprisingly, purified ORC, recombinant Cdc6, and an ATP regenerating system incubated with DNA and purified baculovirus Xcyclin E/XCdk2 could reconstitute a large fraction of cyclin E–Cdk2 binding to the DNA template (Fig. 3 C). If ORC and Cdc6 were not added, no cyclin E–Cdk2 was recruited to DNA. Thus, in phase one, these two preinitiation factors can function as the cyclin E–Cdk2 receptor on purified DNA.

Recombinant Cdc6 Binds Directly to the Hypophosphorylated Forms of Cyclin E–Cdk2 In Vitro

Recent reports have suggested that human Cdc6 binds efficiently to human cyclin A but only weakly to cyclin E (Saha et al., 1998; Petersen et al., 1999). However, we find *Xenopus* ORC and Cdc6 are sufficient to bind cyclin E–Cdk2 to DNA. Because ORC recruits Cdc6 (Coleman et al., 1996), we tested whether XCdc6 could bind directly to the *Xenopus* cyclin E–Cdk2 complex. When bacterially expressed GST–XCdc6 was incubated together with bacu-

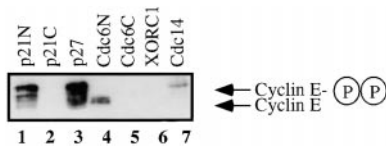


Figure 4. Purified cyclin E-Cdk2 binds directly to Cdc6. Baculovirus-expressed cyclin E-Cdk2 was incubated for 30 min with an energy regeneration system and purified GST fusion proteins including GST-p21N₁₋₉₀ (lane 1), GST-p21C₈₇₋₁₆₄ (lane 2), GST-p27 (lane 3), GST-Cdc6N₂₋₁₆₈ (lane 4), GST-Cdc6C₁₆₉₋₅₅₄ (lane 5), GST-XORC1 (lane 6), or GST-hCdc14 (lane 7). Reactions were diluted in IP buffer and bound to glutathione-agarose beads. Beads were washed and resolved by SDS-PAGE, and cyclin E was visualized by Western blotting.

lovirus-expressed Xcyclin E-Cdk2, the two proteins efficiently coprecipitated. Addition of an energy regenerating system appeared to stimulate binding but was clearly not essential. Furthermore, the NH₂-terminal half of the Cdc6 protein, which contains all three Cy-RXL motifs (see below), was sufficient for this interaction, whereas the COOH-terminal portion was not (Fig. 4).

Although the specific Cdk inhibitors, p21 and p27, could bind all of the various phosphorylated forms of cyclin E, the NH₂ terminus of Cdc6 preferentially bound the lower (hypophosphorylated) form (Fig. 4), the same form that binds most readily to chromatin. As a control for this type of phosphorylation specificity, we also showed that the cell cycle phosphatase Cdc14, which specifically dephosphorylates mitotically phosphorylated Cdk2 and Cdc2 substrates (Kaiser, B.K., C. Swanson, L. Furstenthal, and P.K. Jackson, manuscript in preparation), binds only the upper hyperphosphorylated forms of cyclin E, likely because Cdc14 binds to the phosphoserine or phosphothreonine moiety of cyclin E before dephosphorylating it. Thus, the interaction of cyclin E-Cdk2 with Cdc6 appears to be inhibited by cyclin E phosphorylation (see below).

The MRAIL Motif of Cyclin E Is Required to Bind Cdc6, Facilitate Chromatin Recruitment, and Initiate DNA Replication

RXL (Cy) motifs in Cdk substrates and inhibitors are thought to bind to the hydrophobic MRAIL motif in cyclins (Adams et al., 1996; Chen et al., 1996; Russo et al., 1996; Schulman et al., 1998). Comparing Cdc6 protein sequences from *Xenopus*, human, and mouse, we noted the conservation of two RXL domains (residues 93-95 and 258-260) in the NH₂-terminal half of the protein, surrounded by consensus Cdk phosphorylation sites, with *Xenopus* containing a third nonconserved RXL motif (residues 165-167). To test whether the interaction between XCdc6 and Xcyclin E is dependent upon an RXL-MRAIL interaction, we first mutagenized the hydrophobic MRAIL domain of the Xcyclin E protein: amino acids M₁₄₃, L₁₄₇, and W₁₅₀, or L₁₈₆ and Q₁₈₇ were mutated to alanine (Fig. 5 A). Unlike the wild-type cyclin E protein, neither mutant bound the inhibitor p21 or the substrate Cdc6 in vitro (Fig. 5 B). Previous studies demonstrated that phosphorylation of RXL-containing cyclin-Cdk substrates require an intact MRAIL sequence in the cyclin, whereas phosphorylation of histone H1 does not (Schulman et al., 1998). We also found that relative to wild type, our cyclin E mutants phosphorylated histone H1 efficiently but were

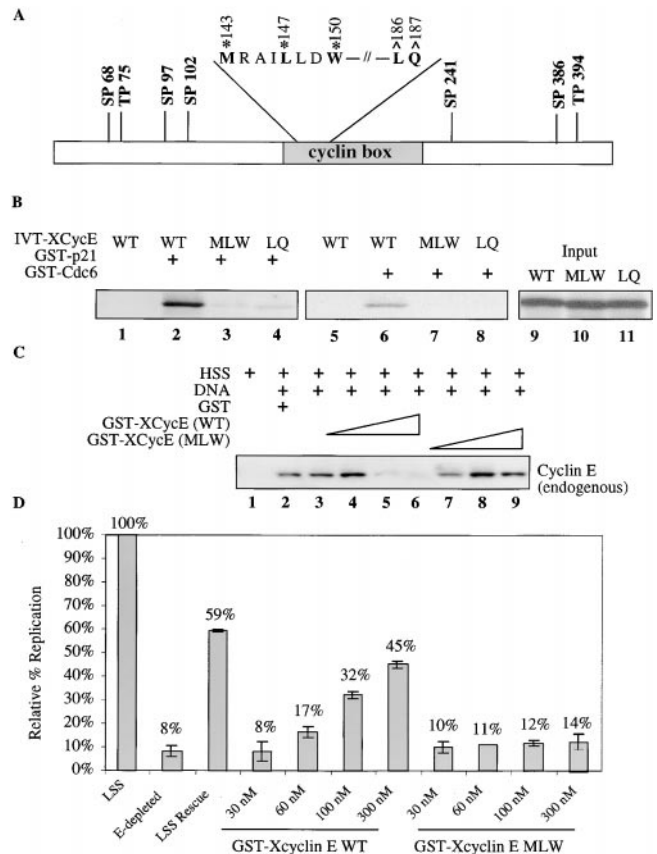


Figure 5. The MRAIL motif of cyclin E is required for binding of cyclin E to Cdc6, recruitment of cyclin E-Cdk2 to DNA, and replication competence. (A) Schematic of the *Xenopus* cyclin E protein. The shaded area indicates the cyclin box. Within this region, the specific amino acids mutated for the MLW mutant (*) and the LQ mutant (^). Putative phosphorylation sites are also depicted, preceded by the amino acid number of the specific serine or threonine residue. (B) Wild-type *Xenopus* cyclin E (lanes 1, 2, 5, and 6), or cyclin E with mutations in the MLW (lanes 3 and 7) or the LQ (lanes 4 and 8) peptide sequences were radiolabeled by in vitro translation in rabbit reticulocyte lysate. The IVT cyclin E variants were added to bacterially expressed GST-p21N (lanes 1-4) or GST-XCdc6 (lanes 5-8), incubated for 30 min, and diluted in IP buffer. GST-p21- and GST-Cdc6-associated cyclin E-Cdk2 was precipitated with glutathione-agarose beads. Beads were washed and resuspended in sample buffer, and proteins were resolved by SDS-PAGE and visualized by autoradiography. Lanes 9-11 show a matched exposure of the amount of input IVT cyclin E used in the binding experiments. (C) HSS was supplemented with buffer (lanes 1 and 2), with 100 nM GST (lane 3) or increasing doses (30, 60, or 100 nM) of wild type (lanes 4-6), or MRAIL mutant (lane 7-9) GST-Xcyclin E. After preincubating the HSS with the GST proteins, λ DNA templates were added to extracts (lanes 2-9), and assembled chromatin was isolated and resolved by SDS-PAGE and blotted for the presence of endogenous cyclin E. The lack of a cyclin E signal in lanes 5 and 6 indicates that wild-type GST-Xcyclin E can effectively compete away chromatin binding of endogenous cyclin E at the indicated concentrations. (D) LSS was immunodepleted with cyclin E antibodies conjugated to protein A-Sepharose beads. Depleted samples were supplemented with undepleted LSS, increasing concentrations of wild type, or MRAIL mutant GST-Xcyclin E, as noted, before the addition of sperm DNA, an energy regenerating system, and [α -³²P]dCTP. Replication was assayed and quantitated in duplicate samples as described in Materials and Methods and plotted as a percentage, normalizing the amount of replication in undepleted LSS to 100%. This corresponds to 1.7 ng/ μ l of new DNA synthesized from the 2.5 ng/ μ l of DNA added.

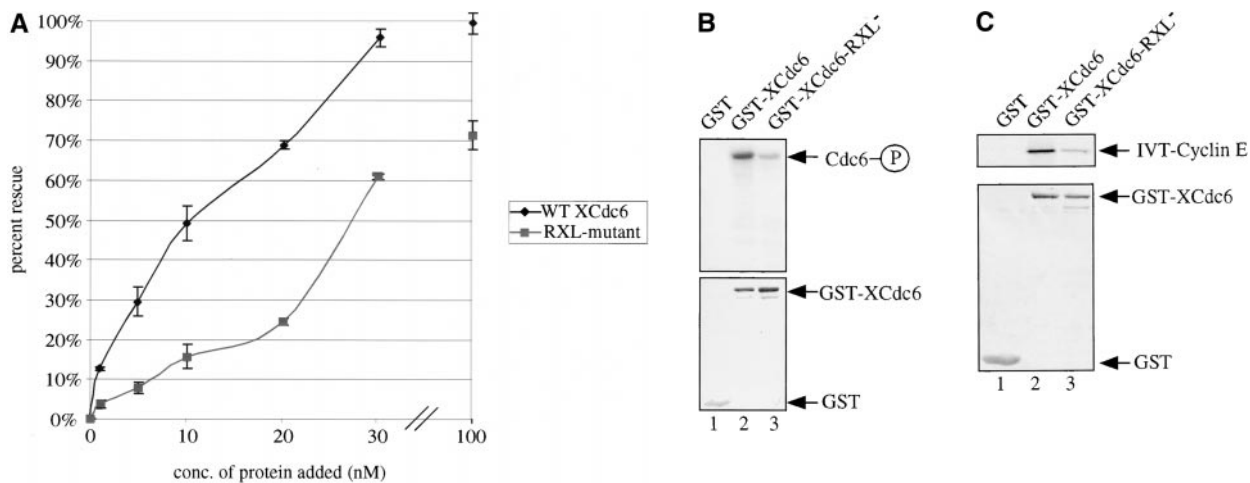


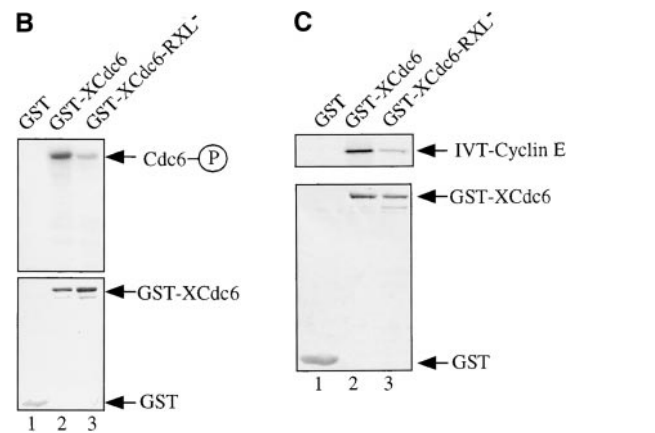
Figure 6. RXL mutants of Cdc6 show a quantitative defect in their ability to bind to cyclin E, to get phosphorylated by cyclin E–Cdk2, and to sustain replication in Cdc6-depleted extract. (A) LSS was immunodepleted with affinity-purified XCdc6 antibodies conjugated to protein A–Sepharose beads. Depleted samples were supplemented with sperm DNA, an energy regenerating system, α [32 P]dCTP, and 1, 5, 10, 20, 30, or 100 nM of either wild-type GST–XCdc6 (◆) or GST–XCdc6 with all three RXL motifs mutated to AXA (■) (see Materials and Methods for mutant description). Replication was quantitated as indicated in Materials and Methods and plotted as a percentage of undepleted extract, normalizing to 100% rescue in mock-depleted extracts and setting 0% replication as the amount of background counts incorporated after depletion. (B) Purified GST (lane 1), wild-type GST–XCdc6 (lane 2), or triple RXL mutant GST–XCdc6 (lane 3) was incubated with purified baculovirus-expressed cyclin E–Cdk2 in the presence of γ [32 P]ATP. Proteins were resolved by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography. Membrane stained with Ponceau S is shown below as a loading control. (C) Purified GST (lane 1), wild-type GST–XCdc6 (lane 2), or triple RXL mutant GST–XCdc6 (lane 3) was incubated with radiolabeled IVT Xcyclin E. After a 30-min incubation, samples were diluted in IP buffer, and GST proteins were precipitated with glutathione–agarose beads and washed. Beads were resuspended in sample buffer, and associated proteins were resolved by SDS-PAGE and visualized by autoradiography. Membrane stained with Ponceau S is shown below as a loading control.

inefficient at phosphorylating Cdc6 (data not shown). Thus, the mutants retain the activity of properly folded proteins towards substrates, but substrate selectivity is altered. Furthermore, wild-type GST–Xcyclin E could compete with the endogenous cyclin E from HSS for binding to chromatin, but the M₁₄₃ L₁₄₇ W₁₅₀ mutant (Fig. 5 C) and the L₁₈₆ Q₁₈₇ mutant (not shown) could not. Therefore, an intact MRAIL domain is necessary to compete for the interaction between cyclin E and chromatin.

Because the MRAIL domain of cyclin E binds Cdc6, we tested whether the MRAIL mutants of cyclin E stimulate replication. We immunodepleted cyclin E from interphase LSS and added back GST fusions of wild-type or MRAIL mutant Xcyclin E. Although the wild-type cyclin protein (30–300 nM) was able to rescue a significant amount of the replication activity in depleted extracts, the mutant protein could not (Fig. 5 D). This suggests that the interaction of cyclin E with Cdc6 is essential for DNA replication, although we cannot exclude the possible importance of other substrates of cyclin E–Cdk2 that require the MRAIL motif. Rescue of the cyclin E depletion with the wild-type GST Xcyclin E protein (45%) was slightly less efficient than rescue with undepleted LSS (59%), which may be due to codepletion of some of the Cdk2 (Jackson et al., 1995), although enough Cdk2 remained to combine with the added cyclin E to rescue a substantial fraction of replication activity.

Cdc6 Containing Mutations in Its RXL Motifs Is Quantitatively Deficient in Binding to Cyclin E, Phosphorylation by Cyclin E–Cdk2, and Sustaining DNA Replication

Because the MRAIL motif of cyclin E is required for DNA replication, we tested whether the RXL (Cy) region



of Cdc6, which likely binds the cyclin E MRAIL motif, is also important for binding to cyclin E and promoting replication. We constructed GST fusion proteins of XCdc6 containing mutations in one, two, or all three RXL domains, including the first RXL motif (R₉₃, L₉₄, L₉₅), the second (R₁₆₅, L₁₆₇), and the third (R₂₅₈, L₂₆₀, mutated to alanine). The triple RXL mutant of Cdc6, which had the most dramatic phenotype, was quantitatively impaired in its ability to bind to cyclin E (Fig. 6 C) and to be phosphorylated by cyclin E–Cdk2 in vitro (Fig. 6 B), although it retained low levels of both respective activities.

When added to Cdc6-depleted *Xenopus* extracts, the triple RXL mutant failed to efficiently rescue replication at and below the concentration of XCdc6 in extract (Fig. 6 A). Adding the triple mutant protein at high levels (>100 nM) rescued up to 70% as well as the wild-type protein; however, at and below concentrations at which the wild-type protein sustained significant rescuing activity, the mutant was 1.5–5-fold less effective. The lower the concentration of the mutant, the more deficient it was at rescuing replication compared with wild-type Cdc6. The degree to which the mutant was able to rescue replication correlated completely with its level of binding to cyclin E and its level of phosphorylation by cyclin E–Cdk2 in vitro. Various combinations of double and single RXL mutants were quantitatively less defective in rescuing replication than the triple mutant; but, the degree of rescue consistently correlated with the number of remaining wild-type RXLs (data not shown). The RXL mutants appear to be otherwise functional, as each bound ORC equivalently to wild-type XCdc6 (data not shown).

Also, we examined a series of Cdc6 NH₂-terminal deletion mutants (see Figure S1, available at <http://>

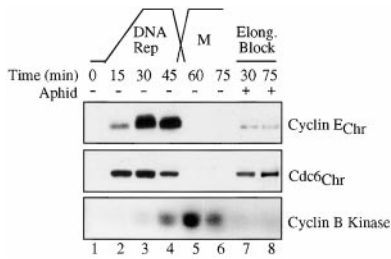


Figure 7. Replication elongation is required for cyclin E accumulation on chromatin. Cycling LSS extracts were incubated with sperm DNA for the indicated times in the absence (lanes 1–6) or the presence (lanes 7 and 8) of aphidicolin (Aphid; 40 μ g/ml) before isolating chromatin templates by sedimentation and resolving chromatin-associated proteins by SDS-PAGE. Top shows Western blots for cyclin E and Cdc6, which remain bound to chromatin in varying amounts throughout DNA replication (DNA rep). Later time points showed no additional assembly of cyclin E onto chromatin in aphidicolin-treated samples. Bottom shows IP kinase assays of samples identical to those above. Anti-cyclin B antibodies conjugated to protein A–Sepharose beads were used to immunoprecipitate cyclin B, and associated kinase activity was assayed by *in vitro* phosphorylation of histone H1 in the presence of γ [³²P]ATP. The peak in cyclin B kinase activity indicates that the extracts are in mitosis (M).

www.jcb.org/cgi/content/full/152/6/1267/DC1). Mutants missing the NH₂-terminal 81 or 108 amino acids of Cdc6 bound cyclin E were efficient cyclin E–Cdk2 substrates *in vitro* and stimulated DNA replication. However, mutants lacking 178 or 251 NH₂-terminal amino acids completely failed to bind cyclin E, be phosphorylated, or stimulate DNA replication. These mutants suggested that additional determinants in the 108–178 amino acid sequence (a region that contains only one RXL) are quantitatively important for cyclin E binding and DNA replication. Each of these truncated Cdc6 proteins bound ORC efficiently, suggesting that they were properly folded to retain other activities. These deletion mutants further support the connection between cyclin E–Cdc6 binding and replication.

Also, we found that an NH₂-terminal fragment of XCdc6 (amino acids 1–258) containing the cyclin E binding region (Fig. 4) inhibited replication at a concentration of \sim 300 nM and completely abrogated replication at \sim 2 μ M (data not shown). This is comparable to the concentrations of p21 that inhibits replication and \sim 3.8 times the concentration of endogenous Cdc6 in extract (80 nM; Coleman et al., 1996). Thus, interfering with the cyclin E–Cdc6 interaction, either by mutation of the RXL motifs in Cdc6, by deletions in the NH₂ terminus, or by addition of Cdc6 fragments that bind cyclin E but do not contain the ORC binding region, suppresses replication. Therefore, the first phase of cyclin E recruitment to chromatin by Cdc6 appears to be essential for DNA replication.

Cyclin E Accumulation on Chromatin Depends on Polymerase Activity

In a second phase, cyclin E continued to accumulate on chromatin throughout replication (Fig. 1 A). Addition of the polymerase α inhibitor, aphidicolin, did not effect the initial binding of cyclin E to chromatin but blocked the subsequent accumulation step (Fig. 7), indicating that polymerase activity is essential for the accumulation of cy-

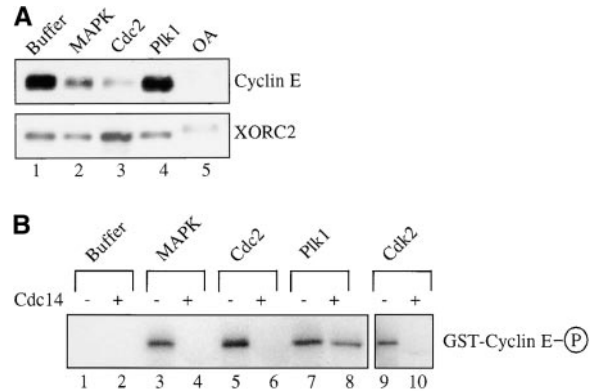


Figure 8. Specific mitotic kinases are capable of phosphorylating cyclin E and displacing cyclin E–Cdk2 from chromatin; Cdc14 can oppose phosphorylation by these kinases. (A) Sperm chromatin assembled in interphase LSS (in the presence of cycloheximide) for 1 h was subsequently treated with buffer (lane 1), 1 U of MAP kinase (lane 2), cyclin B–Cdc2 (lane 3), Plk1 (lane 4), or 10 μ M okadaic acid (lane 5) for 10 min. Chromatin was extracted, and associated proteins were resolved by SDS-PAGE and Western blotted for the presence of cyclin E or ORC. (B) Purified GST–Xcyclin E was incubated with buffer (lanes 1 and 2), MAP kinase (lanes 3 and 4), cyclin B–Cdc2 (lanes 5 and 6), Plk1 (lanes 7 and 8), or cyclin E–Cdk2 (lane 9–10) in the presence of γ [³²P]ATP. After 30 min, 2 μ M GST–Cdc14 was added to indicated samples (lanes 2, 4, 6, 8, and 10), and all samples were incubated for an additional 30 min. Reactions were resolved by SDS-PAGE, and phosphorylated GST–cyclin E was visualized by autoradiography.

clin E–Cdk2 on chromatin. Addition of aphidicolin had no effect on the level of Cdc6 (Fig. 7) or ORC (not shown) bound to chromatin.

MAP Kinase and Cyclin B–Cdc2, but Not Plk1, Dissociate Cyclin E–Cdk2 from Chromatin

To further understand the importance of cyclin E–Cdk2 recruitment to chromatin, we wanted to define requirements for the mitotic displacement of cyclin E from chromatin (the third phase). This displacement (Figs. 1 A and 7) is consistent with previous data showing that Cdc6 is displaced from mitotic chromatin and our data showing that Cdc6 is required for cyclin E binding. However, we also noted that hyperphosphorylated cyclin E, as seen in mitotic extracts (see below), does not bind to Cdc6 (Fig. 4).

To determine if any of several essential mitotic kinases were capable of phosphorylating cyclin E and displacing the cyclin E–Cdk2 complex from chromatin, we treated chromatin assembled in interphase LSS extracts with cyclin B–Cdc2, MAP kinase, or the polo-like kinase (Plk1) (Murray and Kirschner, 1989; Lane and Nigg, 1996; Guadagno and Ferrell, 1998) and isolated assembled chromatin. Although treatment with Plk1 had no effect, cyclin B–Cdc2 efficiently removed cyclin E–Cdk2 from chromatin (Fig. 8 A). Addition of MAP kinase could also displace the majority of cyclin E–Cdk2 from chromatin, but less efficiently (Fig. 8 A). Both cyclin B–Cdc2 and MAP kinase phosphorylated purified GST–cyclin E *in vitro* (Fig. 8 B), suggesting that the effect on cyclin E may be direct. Plk1 also phosphorylated GST–cyclin E *in vitro* (Fig. 8 B), but the significance of this remains unclear. The Cdc14 phos-

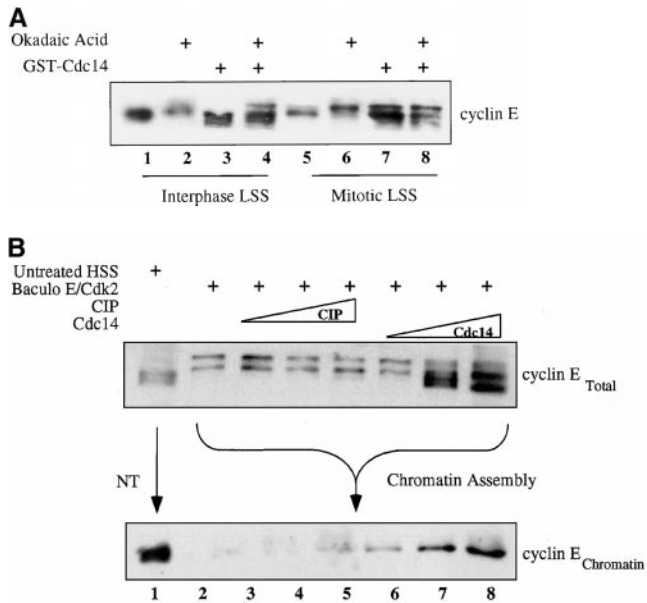


Figure 9. Cdc14 reverses the inability of mitotic hyperphosphorylated cyclin E to bind to chromatin. (A) Interphase extract (lanes 1–4) or mitotic extract stabilized by the addition of nondestructible cyclin B (lanes 5–8) was supplemented with buffer (lanes 1 and 4), 10 μ M okadaic acid (OA; lanes 2 and 6), 1 μ M GST-Cdc14 (lanes 3 and 7), or both okadaic acid and Cdc14 (lanes 4 and 8) and incubated at 23°C for 30 min. Reactions were stopped by adding sample buffer, proteins were resolved by SDS-PAGE, and Western blots were performed with cyclin E antibodies to detect the various phosphorylated forms of cyclin E. (B) Baculovirus-expressed *Xenopus* cyclin E-Cdk2 in an autohyperphosphorylated form was mixed with buffer (lane 2), increasing concentrations of the CIP phosphatase (lanes 3–5), or increasing concentrations of GST-Cdc14 (lanes 6–8) for 30 min. Untreated HSS (lane 1) and treated samples were resolved by SDS-PAGE and analyzed by Western blotting with antibodies to cyclin E (top). The samples in lanes 2–8 were incubated with λ DNA templates and a small amount of HSS (bottom). Assembled chromatin was isolated by sedimentation, and proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-cyclin E antibodies. The sample in lane 1 is HSS that was not treated (NT).

phatase was capable of reversing the phosphorylation of cyclin E by both Cdc2 and MAP kinase but not by Plk1 (Fig. 8 B), indicating that Plk1 likely phosphorylates cyclin E on different sites from Cdc2 and MAP kinase.

The Mitotic Phosphorylation of Cyclin E That Blocks Chromatin Recruitment Can Be Reversed by the Cdc14 Phosphatase

Previously, we had found that during mitosis cyclin E-Cdk2 is hyperphosphorylated on the cyclin and is approximately threefold increased in activity. This mitotic hyperphosphorylation is inhibited by the Cdk inhibitor p21, indicating that this phosphorylation is Cdk-dependent, likely by one of the mitotic Cdk activities in eggs: cyclin A-Cdc2, cyclin B-Cdc2, or cyclin E-Cdk2 (P.K. Jackson, unpublished data). Cyclin E-Cdk2 can also autophosphorylate on the cyclin. To correlate the changes in the phosphorylation of cyclin E with mitotic events, we examined the mobility of cyclin E from mitotic or interphase extracts by SDS-PAGE, visualized by Western blotting.

Cyclin E was present in at least two forms in interphase extract. Addition of the phosphatase 2A inhibitor and mitotic inducer, okadaic acid, (Goris et al., 1989), resulted in hyperphosphorylation of cyclin E, as did addition of a nondestructible form of cyclin B. This phosphorylation was reversed by the mitotic phosphatase Cdc14 (Fig. 9 A). Cdc14 has been found to be important for the exit from mitosis and appears to function by dephosphorylating substrates of cyclins E, A, and B (Kaiser, B.K., C. Swanson, L. Furstenthal, and P.K. Jackson, manuscript in preparation). In vitro, Cdc14 can directly dephosphorylate cyclin E that has been previously phosphorylated by MAP kinase, cyclin B-Cdc2, or cyclin E-Cdk2 autophosphorylation, but not Plk1 (Fig. 8 B).

To test whether phosphorylation of cyclin E affected chromatin binding, we prepared uniformly autophosphorylated cyclin E-Cdk2 (Materials and Methods). We observed that hyperphosphorylated cyclin E-Cdk2 was unable to bind to chromatin, even in the presence of HSS (Fig. 9 B). Because Cdc14 can reverse the mitotic phosphorylation of cyclin E in vitro (Fig. 8 B) and because Cdc14 is required for mitotic exit in yeast (Wood and Hartwell, 1982; Visintin et al., 1998), we tested whether Cdc14 would also promote the binding of hyperphosphorylated cyclin E to chromatin. We treated hyperphosphorylated cyclin E-Cdk2 with the Cdc14 phosphatase or with calf intestinal phosphatase (CIP) as a control. Only Cdc14, and not CIP, was able to dephosphorylate cyclin E (Fig. 9 B). The collapse of bands seen in Fig. 9 B upon treatment of cyclin E with Cdc14 corresponds to dephosphorylation of cyclin E. When the phosphatase-treated fractions of cyclin E-Cdk2 were tested in the chromatin assembly assay, only the Cdc14-treated dephosphorylated cyclin E bound to chromatin, whereas untreated and CIP-treated fractions did not (Fig. 9 B). Thus, Cdc14 or a similar phosphatase may dephosphorylate mitotic cyclin E-Cdk2 to allow chromatin binding after mitosis, setting up a new round of DNA replication.

Discussion

Cyclin E-Cdk2 Binds to a Saturable Chromatin Receptor Composed of ORC, Cdc6, and Possibly Other Factor(s)

We have detailed the requirements and cell cycle behavior of the cyclin E-Cdk2-chromatin interaction. A previous study did not see cyclin E associating with chromatin (Hua et al., 1997). This study showed that in buffers containing the detergent Triton X-100 and lacking chromatin stabilizing factors such as spermine, spermidine, and ATP, the ORC complex remained bound to chromatin, but no cyclin E was observed. Under these specific conditions, we find that cyclin E-Cdk2, and both Cdc6 and MCM3, are stripped from chromatin (Furstenthal, L., and P.K. Jackson, unpublished results). However, our data is consistent with a previous immunofluorescence study, which observed that cyclin E colocalizes with decondensed, but not mitotic, chromatin (Chevalier et al., 1996).

There are several reasons why we observe modest levels of cyclin E binding to chromatin in the absence of the nucleus and why we need to add exogenous cyclin E-Cdk2 to see a strong signal in our HSS chromatin binding assay. Although the major constituents of the cyclin E chromatin

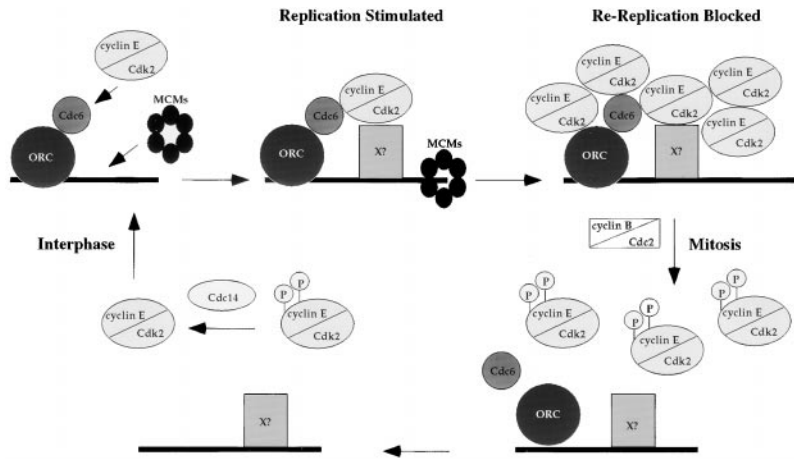


Figure 10. Model of the cell cycle-regulated association of cyclin E-Cdk2 with chromatin and its effects on DNA replication and rereplication control. In a first phase, cyclin E-Cdk2 is recruited to origins of DNA replication by ORC, Cdc6, and possibly an unknown factor (X?). In this conformation, with MCMs bound, DNA replication is initiated. In a second phase, dependent on the progression of replication forks, multiple molecules of cyclin E accumulate on chromatin, blocking re-replication. In a final phase, cyclin E is hyperphosphorylated by cyclin B-Cdc2 and stripped from chromatin in mitosis. Rebinding of cyclin E to chromatin in interphase is possible only after dephosphorylation by Cdc14 or a related phosphatase (Discussion).

receptor, ORC and Cdc6, bind to chromatin with high affinity in membrane-free extracts, we find that nuclear import, or a step subsequent to it, is required for cyclin E-Cdk2 to bind chromatin efficiently. Hua and colleagues (1997) have shown that cyclin E is concentrated 200-fold in the nucleus (to $>5 \mu\text{M}$) upon nuclear assembly (Hua et al., 1997). The cyclin E-Cdc6 interaction appears to be of sufficiently low affinity to require the active concentration of cyclin E to drive its chromatin association. We find that cyclin E binds to Cdc6 with much lower apparent affinity than to p21 (Figs. 4 and 5 B). Additionally, because cyclin E directs ubiquitylation and destruction of its bound inhibitor p27^{Xic1} only on chromatin (Furstenthal, L., C. Swanson, B.K. Kaiser, and P.K. Jackson, manuscript submitted for publication) after nuclear accumulation of the cyclin E-Cdk2-Xic1 complex (Swanson et al., 2000), an SCF activity important for p27^{Xic1} destruction may need to be associated with cyclin E-Cdk2 to help recruit or stabilize the cyclin E complex on chromatin. SCF activity towards p27^{Xic1} requires the prior assembly of ORC, Cdc6, and MCM proteins onto chromatin (Furstenthal, L., C. Swanson, B.K. Kaiser, and P.K. Jackson, manuscript submitted for publication). Possibly, Cdc7 is also required, resulting in a sequential link whereby Cdc7 acts before Cdk2 activation to trigger replication initiation, as was recently observed (Jares and Blow, 2000; Walter, 2000).

ORC-Cdc6 is one, but may not be the only, receptor for cyclin E-Cdk2 on chromatin. Our reconstituted chromatin binding reaction allowed us to show that ORC and Cdc6 are required for the first phase of cyclin E-Cdk2 recruitment to chromatin. Residual cyclin E-Cdk2 binding to chromatin in the absence of ORC or Cdc6 suggests that there may be other yet unidentified factor(s) that recruit cyclin E-Cdk2 to chromatin.

By quantitating the amount of cyclin E bound to chromatin during the cell cycle, we gain possible insight into cyclin E's multiple roles in promoting initiation, preventing rereplication, and allowing origin resetting. During the first phase, we see binding of cyclin E-Cdk2 to chromatin at $\sim 100 \text{ nM}$, just above the concentration of cyclin E in interphase cytosol ($\sim 60 \text{ nM}$; Hua et al., 1997). This explains why we see only minimal binding of cyclin E to DNA in HSS and why cyclin E-Cdk2 must be concentrated in the nucleus to facilitate full binding. The chromatin receptor for cyclin E appears saturated when exogenous cyclin E-Cdk2 is added to HSS at $\sim 1 \mu\text{M}$, approximately the con-

centration of cyclin E-Cdk2 found in the nucleus soon after nuclear formation. This level of cyclin E-Cdk2 binding to chromatin corresponds to about one cyclin E molecule per origin during early replication. As replication proceeds, cyclin E-Cdk2 is deposited on chromatin, dependent on the action of polymerase. We find ~ 5 – 10 -fold more cyclin E binds by the end of replication (Materials and Methods for calculations). This wide range of cyclin E-Cdk2 binding, beginning with low binding in phase one before origins have fired, and increasing to high levels throughout phase two as replication proceeds, provides a potential mechanism for the observations that cyclin E both promotes initiation and prevents rereplication. The chromatin substrates of cyclin E-Cdk2 that become phosphorylated to initiate replication or to block rereplication remain unknown, but ORC and Cdc6 themselves are reasonable candidates (see below).

Cyclin E Uses Its MRAIL Motif to Bind Cdc6 NH₂-terminal/RXL Sequences, an Interaction Important for DNA Replication

Our data suggest that the interaction between cyclin E and Cdc6 on chromatin is essential for DNA replication. Work in yeast has also shown that the NH₂-terminal 47 amino acids of Cdc6 interact with the Cdk complex that promotes initiation in *Saccharomyces cerevisiae*, Clb5-Cdc28. However, the Cdc6-Cdc28 interaction in *S. cerevisiae* appears to be a complicated one, required at physiological levels of Cdc6, but not when the Cdc6 protein, missing the NH₂-terminal amino acid minimal binding domain for cyclin-Cdc28, is overexpressed (Elsasser et al., 1996). This work complements our study, suggesting that the strength of the Cdc6-Cdk interaction is concentration-dependent and likely indicating that a domain beyond the NH₂ terminus of *S. cerevisiae* cyclin-Cdc6p is also involved in binding Cdc6 to Cdk complexes, but with lower affinity. Although a canonical Cy-RXL motif of *S. cerevisiae* Cdc6 lies very close to the NH₂ terminus, a second RXL motif can be found in the middle of the protein.

NH₂-terminal deletions of Cdc6, and mutations in the RXL motif cause strong or moderate loss of cyclin E-Cdk2 binding and a parallel loss in the ability of these Cdc6 variants to stimulate DNA replication. There may be important determinants for cyclin E and Cdc6 to interact in residues 108–178, independent of the Cdc6 RXL motifs.

Also, our work suggests a correlation between phosphorylation of Cdc6 and DNA replication. In yeast, phosphorylation of Cdc6 has been shown to play a role in its destruction (Elsasser et al., 1999). In human cells, cyclin E–Cdk2 phosphorylates Cdc6 in vitro and in vivo at three sites in the Cdc6 NH₂ terminus, close to the RXL motif, and phosphorylation by Cdks appears to control the localization of Cdc6 (Saha et al., 1998; Jiang et al., 1999). In studying the various combinations of RXL mutants in *Xenopus* Cdc6, we noticed a strong correlation between the degree of in vitro phosphorylation of the XCdc6 mutants by cyclin E–Cdk2 and the amount of DNA replication sustained by each mutant in Cdc6-depleted extract. A recent report found that an unphosphorylatable mutant of XCdc6 supports a single round of DNA replication (Pelizon et al., 2000). Nonetheless, the quintuple serine mutant used in the study by Pelizon et al. (2000) still contains intact threonine residues that are part of Cdk consensus sequences, and may therefore sustain a low but sufficient level of phosphorylation to promote replication. However, mutation of the five serine residues does prevent nuclear export of Cdc6. Thus, phosphorylation of Cdc6 by cyclin E–Cdk2 (or in human cells, cyclin A–Cdk2) may occur after initiation, causing Cdc6 to exit the nucleus to prevent rereplication. This is consistent with our model, wherein a build-up of cyclin E–Cdk2 on chromatin, coincident with the movement of polymerase, could allow concentration-dependent phosphorylation of Cdc6 on chromatin to dislodge or promote destruction of the Cdc6 protein. Our results show that Cdc6 must recruit cyclin E–Cdk2 to chromatin for efficient replication. The results of Pelizon et al. (2000) argue that Cdc6 phosphorylation by cyclin E–Cdk2 is not positively required for replication. Together, these data suggest that the interaction between cyclin E–Cdk2 and Cdc6 may be biochemically distinct from a kinase–substrate interaction; instead, Cdc6 may serve to recruit or organize cyclin E–Cdk2's ability to direct downstream events of origin unwinding.

In human USO2 cells, cyclin A, rather than cyclin E, mediates the majority of Cdc6 phosphorylation by Cdks (Petersen et al., 1999). This may simply reflect differences between human somatic cells and amphibian eggs. Note that in human cells cyclin A is a primary partner of Cdk2, whereas in *Xenopus* eggs ~90% of the Cdk2 is associated with cyclin E (Jackson et al., 1995), and cyclin A is complexed with Cdc2 (Minshull et al., 1989).

Mitotic Regulation of the Cyclin E–Cdk2 Chromatin Association May Be an Important Mechanism in Rereplication Control

We found that mitotic cyclin E hyperphosphorylation apparently causes the cyclin E–Cdk2 complex to be removed from chromatin. Several arguments suggest that cyclin B–Cdc2 directly phosphorylates cyclin E in mitosis to cause its displacement from chromatin. First, cyclin E disappears from chromatin after replication is complete (Figs. 1 A and 7), when high levels of cyclin B–Cdc2 activity indicate that the extracts are in mitosis. Second, cyclin E is unable to associate with chromatin assembled in CSF-arrested mitotic extracts in the absence of calcium (Furstenthal, L., and P.K. Jackson, unpublished data) when cyclin B kinase activity is high. Third, the dissociation of cyclin E–Cdk2

from chromatin assembled in cycling extracts can be blocked by cycloheximide addition, which prevents cyclin B synthesis and entry into mitosis (Fig. 1 A). Finally, addition of cyclin B–Cdc2 to fully assembled interphase chromatin removes cyclin E from the chromatin template (Fig. 8 A). The ability of cyclin B–Cdc2 to phosphorylate recombinant cyclin E in vitro (Fig. 8 B) suggests that this effect is direct, rather than an indirect result of inducing mitosis. MAP kinase addition can also dissociate cyclin E from chromatin, although less efficiently than cyclin B–Cdc2 (Fig. 8 A). This result may indicate that MAP kinase is important for keeping cyclin E from rebinding to chromatin in late mitosis, when MAP kinase functions to maintain the mitotic state after Cdc2 inactivation (Guadagno and Ferrell, 1998). It has been observed that the activity of cyclin E–Cdk2 is approximately threefold higher in mitosis (Fang and Newport, 1991; Jackson et al., 1995). Thus, it is possible that cyclin E–Cdk2 autophosphorylation contributes to its mitotic displacement. The essential mitotic kinase Plk1, a homologue of *Drosophila* polo, does not appear to affect cyclin E chromatin binding.

Dephosphorylation of cyclin E by Cdc14 reverses the effects of the mitotic kinases and promotes cyclin E–Cdk2 binding to chromatin. In budding yeast, Cdc14 plays an essential role in the exit from mitosis (Visintin et al., 1998), in part by reversing the mitotic phosphorylation of Cdk substrates. We have found that Cdc14 plays a similar role in vertebrates (Kaiser, B.K., and P.K. Jackson, unpublished data). Thus, the dephosphorylation of cyclin E by Cdc14 after mitosis may provide one explanation for how Cdc14 promotes mitotic exit. However, Cdc14 may not be the only phosphatase capable of increasing the amount of cyclin E on chromatin. Phosphatase 1 is also capable of dephosphorylating *Xenopus* cyclin E in vitro (Rempel et al., 1995), and is also important for progression out of mitosis (Maller, 1994).

The regulation of cyclin E–Cdk2 chromatin association by phosphorylation may help explain how cyclin E mediates rereplication control. Oscillations in the level of cyclin E–Cdk2 are required for *Drosophila* endocycles, as constitutive expression of cyclin E in *Drosophila* salivary glands inhibits cell growth and further rounds of DNA replication (Follette et al., 1998). A similar phenomenon was reported in *Xenopus* extracts, which are unable to replicate in the presence of high levels of cyclin E–Cdk2 (Hua et al., 1997). We show that, in phase two, cyclin E accumulates on chromatin as replication progresses (Fig. 1 A) and that chromatin accumulation of cyclin E can be blocked at stage one levels by addition of the polymerase α elongation inhibitor, aphidicolin (Fig. 7). Our data is thus consistent with cyclin E–Cdk2 playing a role in both initiation and rereplication control, since it appears to bind additional chromatin receptor(s) as replication progresses and to be stripped from chromatin via phosphorylation by Cdc2 and/or MAP kinase in mitosis. In the next cell cycle, a permissive state for cyclin E–Cdk2–chromatin binding may be reestablished by Cdc14 dephosphorylation of cyclin E upon the exit from mitosis and entry into G1 (Fig. 10)

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References

- Adams, P.D., W.R. Sellers, S.K. Sharma, A.D. Wu, C.M. Nalin, and W.G. Kaelin, Jr. 1996. Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol. Cell. Biol.* 16: 6623–6633.
- Bell, S.P., and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature.* 357:128–134.
- Carpenter, P.B., P.R. Mueller, and W.G. Dunphy. 1996. Role for a *Xenopus* Orc2-related protein in controlling DNA replication. *Nature.* 379:357–360.
- Chen, J., P.K. Jackson, M.W. Kirschner, and A. Dutta. 1995. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature.* 374:386–388.
- Chen, J., P. Saha, S. Kornbluth, B.D. Dynlacht, and A. Dutta. 1996. Cyclin-binding motifs are essential for the function of p21CIP1. *Mol. Cell. Biol.* 16: 4673–4682.
- Chevalier, S., A. Couturier, I. Chartrain, R. Le Guellec, C. Beckhelling, K. Le Guellec, M. Philippe, and C.C. Ford. 1996. *Xenopus* cyclin E, a nuclear phosphoprotein, accumulates when oocytes gain the ability to initiate DNA replication. *J. Cell Sci.* 109:1173–1184.
- Chong, J.P., H.M. Mahubani, C.Y. Khoo, and J.J. Blow. 1995. Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature.* 375:418–421.
- Coleman, T.R., P.B. Carpenter, and W.G. Dunphy. 1996. The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell.* 87:53–63.
- Elsasser, S., F. Lou, B. Wang, J.L. Campbell, and A. Jong. 1996. Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol. Biol. Cell.* 7:1723–1735.
- Elsasser, S., Y. Chi, P. Yang, and J.L. Campbell. 1999. Phosphorylation controls timing of Cdc6p destruction: a biochemical analysis. *Mol. Biol. Cell.* 10: 3263–3277.
- Fang, F., and J.W. Newport. 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell.* 66:731–742.
- Follette, P.J., R.J. Duronio, and P.H. O'Farrell. 1998. Fluctuations in cyclin E levels are required for multiple rounds of endocycle S phase in *Drosophila*. *Curr. Biol.* 8:235–238.
- Goris, J., J. Hermann, P. Hendrix, R. Ozon, and W. Merlevede. 1989. Okadaic acid, a specific protein phosphatase inhibitor, induces maturation and MPF formation in *Xenopus laevis* oocytes. *FEBS Lett.* 245:91–94.
- Guadagno, T.M., and J.E. Ferrell, Jr. 1998. Requirement for MAPK activation for normal mitotic progression in *Xenopus* egg extracts. *Science.* 282:1312–1315.
- Hua, X.H., H. Yan, and J. Newport. 1997. A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J. Cell Biol.* 137: 183–192.
- Jackson, P.K., S. Chevalier, M. Philippe, and M.W. Kirschner. 1995. Early events in DNA replication require cyclin E and are blocked by p21CIP1. *J. Cell Biol.* 130:755–769.
- Jallepalli, P.V., G.W. Brown, M. Muzi-Falconi, D. Tien, and T.J. Kelly. 1997. Regulation of the replication initiator protein p53cdcl8 by CDK phosphorylation. *Genes Dev.* 11:2767–2779.
- Jares, P., and J.J. Blow. 2000. *Xenopus* cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. *Genes Dev.* 14:1528–1540.
- Jiang, W., N.J. Wells, and T. Hunter. 1999. Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. *Proc. Natl. Acad. Sci. USA.* 96: 6193–6198.
- Knoblich, J.A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C.F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell.* 77:107–120.
- Kubota, Y., S. Mimura, S. Nishimoto, H. Takisawa, and H. Nojima. 1995. Identification of the yeast MCM3-related protein as a component of *Xenopus* DNA replication licensing factor. *Cell.* 81:601–609.
- Lane, H.A., and E.A. Nigg. 1996. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J. Cell Biol.* 135:1701–1713.
- Lee, T.H., M.J. Solomon, M.C. Mumby, and M.W. Kirschner. 1991. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell.* 64:415–423.
- Liang, C., M. Weinreich, and B. Stillman. 1995. ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell.* 81:667–676.
- Maller, J. 1994. Biochemistry of cell cycle checkpoints at the G2/M and metaphase/anaphase transitions. *Semin. Dev. Biol.* 5:183–190.
- Martinez-Campa, C., N.A. Kent, and J. Mellor. 1997. Rapid isolation of yeast plasmids as native chromatin. *Nucleic Acids Res.* 25:1872–1873.
- Minshull, J., J. Pines, R. Golsteyn, N. Standart, S. Mackie, A. Colman, J. Blow, J.V. Ruderman, M. Wu, and T. Hunt. 1989. The role of cyclin synthesis, modification and destruction in the control of cell division. *J. Cell Sci.* 12(Suppl.):77–97.
- Murray, A.W., and M.W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. *Nature.* 339:275–280.
- Murray, A.W., M.J. Solomon, and M.W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature.* 339:280–286.
- Pelizon, C., M.A. Madine, P. Rowmanowski, and R.A. Laskey. 2000. Unphosphorylatable mutants of Cdc6 disrupt its nuclear export but still support DNA replication once per cell cycle. *Genes Dev.* 14:2526–2533.
- Petersen, B.O., J. Lukas, C.S. Sørensen, J. Bartek, and K. Helin. 1999. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:396–410.
- Rao, H., and B. Stillman. 1995. The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proc. Natl. Acad. Sci. USA.* 92:2224–2228.
- Rempel, R.E., S.B. Sleight, and J.L. Maller. 1995. Maternal *Xenopus* Cdk2-cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G1 phase. *J. Biol. Chem.* 270:6843–6855.
- Romanowski, P., M.A. Madine, A. Rowles, J.J. Blow, and R.A. Laskey. 1996. The *Xenopus* origin recognition complex is essential for DNA replication and MCM binding to chromatin. *Curr. Biol.* 6:1416–1425.
- Rowles, A., J.P. Chong, L. Brown, M. Howell, G.I. Evan, and J.J. Blow. 1996. Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell.* 87:287–296.
- Russo, A.A., P.D. Jeffrey, A.K. Patten, J. Massagué, and N.P. Pavletich. 1996. Crystal structure of the p27Kip1 cyclin-dependent kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature.* 382:325–331.
- Saha, P., J. Chen, K.C. Thome, S.J. Lawlis, Z.H. Hou, M. Hendricks, J.D. Parvin, and A. Dutta. 1998. Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol. Cell. Biol.* 18:2758–2767.
- Schulman, B.A., D.L. Lindstrom, and E. Harlow. 1998. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc. Natl. Acad. Sci. USA.* 95:10453–10458.
- Strausfeld, U.P., M. Howell, R. Rempel, J.L. Maller, T. Hunt, and J.J. Blow. 1994. Cip1 blocks the initiation of DNA replication in *Xenopus* extracts by inhibition of cyclin-dependent kinases. *Curr. Biol.* 4:876–883.
- Strausfeld, U.P., M. Howell, P. Descombes, S. Chevalier, R.E. Rempel, J. Adamczewski, J.L. Maller, T. Hunt, and J.J. Blow. 1996. Both cyclin A and cyclin E have S-phase promoting (SPF) activity in *Xenopus* egg extracts. *J. Cell Sci.* 109:1555–1563.
- Swanson, C., J. Ross, and P.K. Jackson. 2000. Nuclear accumulation of cyclin E-Cdk2 triggers a concentration-dependent switch for the destruction of p27Xic1. *Proc. Natl. Acad. Sci. USA.* 97:7796–7801.
- Swedlow, J.R., and T. Hirano. 1996. Fuzzy sequences, specific attachments? Chromosome dynamics. *Curr. Biol.* 6:544–547.
- Tye, B.-K. 1994. The MCM2-3-5 proteins: are they replication licensing factors? *Trends Cell Biol.* 4:160–166.
- Visintin, R., K. Craig, E.S. Hwang, S. Prinz, M. Tyers, and A. Amon. 1998. The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. *Mol. Cell.* 2:709–718.
- Walter, J.C. 2000. Evidence for sequential action of Cdc7 and Cdk2 protein kinases during initiation of DNA replication in *Xenopus* extracts. *J. Biol. Chem.* 275:39773–39778.
- Walter, J., and J.W. Newport. 1997. Regulation of replicon size in *Xenopus* egg extracts. *Science.* 275:993–995.
- Walter, J., L. Sun, and J. Newport. 1998. Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell.* 1:519–529.
- Weiss, A., A. Herzig, H. Jacobs, and C.F. Lehner. 1998. Continuous Cyclin E expression inhibits progression through endoreduplication cycles in *Drosophila*. *Curr. Biol.* 8:239–242.
- Williams, R.S., R.V. Shohet, and B. Stillman. 1997. A human protein related to yeast Cdc6p. *Proc. Natl. Acad. Sci. USA.* 94:142–147.
- Wood, J.S., and L.H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 94:718–726.
- Yan, H., and J. Newport. 1995. An analysis of the regulation of DNA synthesis by cdk2, Cip1, and licensing factor. *J. Cell Biol.* 129:1–15.
- Yan, H., S. Gibson, and B.K. Tye. 1991. Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. *Genes Dev.* 5:944–957.
- Zhao, J., B. Dynlacht, T. Imai, T. Hori, and E. Harlow. 1998. Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. *Genes Dev.* 12:456–461.