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Xenopus Mcm10 Binds to Origins of DNA Replication after Mcm2-7 and Stimulates Origin Binding of Cdc45

James A. Wohlschlegel,[1] Suman K. Dhar,[1] Tatyana A. Prokhorova,[2] Anindya Dutta,[1,3] and Johannes C. Walter[2,3]
[1]Department of Pathology
Brigham and Women's Hospital
75 Francis Street
[2]Department of Biological Chemistry
and Molecular Pharmacology
240 Longwood Avenue
Harvard Medical School
Boston, Massachusetts 02115

Summary

Current models suggest that the replication initiation factor Mcm10 is required for association of Mcm2-7 with origins of replication to generate the prereplicative complex (pre-RC). Here we report that *Xenopus* Mcm10 (XMcm10) is not required for origin binding of XMcm2-7. Instead, the chromatin binding of XMcm10 at the onset of DNA replication requires chromatin-bound XMcm2-7, and it is independent of Cdk2 and Cdc7. In the absence of XMcm10, XCdc45 binding, XRPA binding, and initiation-dependent plasmid supercoiling are blocked. Therefore, XMcm10 performs its function after pre-RC assembly and before origin unwinding. As one of the earliest known pre-RC activation steps, chromatin binding of XMcm10 is an attractive target for regulation by cell cycle checkpoints.

Introduction

The initiation of eukaryotic DNA replication is a highly coordinated process governed by the regulated assembly and disassembly of multiple macromolecular protein complexes (reviewed in Dutta and Bell, 1997; Kelly and Brown, 2000). Origins of replication are first recognized and bound by a six-subunit origin recognition complex (ORC). In the G_1 phase of the cell cycle, Cdc6, Cdt1, and Mcm2-7 are recruited to replication origins in an ORC-dependent process to form a prereplicative complex (pre-RC) (Maiorano et al., 2000; Nishitani et al., 2000). Current evidence suggests that the Mcm2-7 complex is the replicative helicase (reviewed in Labib and Diffley, 2001). Upon entry into S phase, DNA replication is initiated by the conversion of pre-RCs into active replication forks. This transformation requires the activity of two families of protein kinases, the Cdc7/Dbf4 kinase and the S phase cyclin-dependent kinases (Cdk), which cooperate to recruit Cdc45 to origins of DNA replication (Zou and Stillman, 2000). While there is good evidence that Cdc7 stimulates initiation by phosphorylating one or more of the Mcm2-7 complex subunits (Sclafani, 2000), the Cdk substrates are unknown. Current models suggest Cdc45 binds to the Mcm2-7 complex (Zou and Stillman, 2000), thereby promoting origin unwinding and the recruitment of replication protein A (RPA) as well as DNA polymerases to the origin (Takisawa et al., 2000; Tanaka and Nasmyth, 1998; Walter and Newport, 2000).

Xenopus egg extracts are a powerful system for studying the biochemical requirements for the initiation of DNA replication. Upon the addition of sperm chromatin to these extracts, a replication-competent nucleus is assembled around the sperm chromatin, and a single round of semiconservative DNA replication occurs (Blow and Laskey, 1986; Newport, 1987). Although replication in *Xenopus* egg extracts initiates without reference to a specific DNA sequence, the biochemical mechanism of initiation in yeast and *Xenopus* is highly conserved (Blow, 2001). Recently, nucleus-independent DNA replication has been achieved in *Xenopus* egg extracts (Walter et al., 1998). This soluble system requires the activity of two different egg extracts. First, sperm chromatin or plasmid DNA is incubated with membrane-free egg cytosol (EC). Because unfertilized *Xenopus* eggs contain a large stockpile of proteins required to undergo many rapid cell divisions, the large volume of egg cytosol contains many proteins that would normally be found in the nucleus, such as ORC, Cdc6, Cdt1, and Mcm2-7. For this reason, egg cytosol supports pre-RC formation on exogenously added DNA. However, the egg cytosol does not contain sufficient levels of Cdk and Cdc7 activities to stimulate initiation from these pre-RCs. As such, egg cytosol mimics the nuclear environment found in the G_1 phase of the cell cycle. To stimulate initiation, a concentrated nucleoplasmic extract (NPE) is added which is prepared from pseudonuclei assembled in crude egg cytoplasm. This extract supplies high levels of Cdc7 (Walter, 2000) and Cdk2 activities (J.C.W., unpublished data) and thereby stimulates initiation from previously assembled pre-RCs. NPE also contains inhibitors that block de novo binding of XMcm2-7 to chromatin. This explains why DNA must be first incubated in egg cytosol and why replication is limited to a single round in this system (Walter et al., 1998). Thus, NPE closely simulates the conditions found during the S phase of the cell cycle.

Some of the replication initiation factors identified in yeast have not yet been characterized in *Xenopus* extracts. One such factor is Mcm10. *MCM10*, also known as *DNA43*, was identified in *S. cerevisiae* in two independent genetic screens for genes required for chromosomal DNA replication and stable plasmid maintenance (Merchant et al., 1997; Solomon et al., 1992). *MCM10* mutants show a dramatic reduction in DNA replication initiation at chromosomal origins and arrest at the nonpermissive temperature with a dumbbell morphology and 2C DNA content, similar to other DNA replication initiation mutants (Merchant et al., 1997). Genetic studies have shown that mutations in *MCM10* can be suppressed by extragenic mutations in *MCM7* and *MCM5* and are synthetically lethal with mutations in *CDC45*, *ORC2*, *ORC5*, *DPB11*, *DNA2*, and genes encoding subunits of DNA polymerase ϵ and DNA polymerase δ

[3]Correspondence: johannes_walter@hms.harvard.edu (J.C.W.), adutta@rics.bwh.harvard.edu (A.D.)

(Homesley et al., 2000; Kawasaki et al., 2000; Liu et al., 2000; Merchant et al., 1997). Results from yeast two-hybrid and in vitro binding experiments suggest that Mcm10 can physically associate with the Mcm2-7 complex (Merchant et al., 1997). It was also recently demonstrated that human Mcm10 can associate with Orc2 when both proteins are overexpressed in cultured cells (Izumi et al., 2000). Mcm10 is bound to a total chromatin fraction in the G_1, S, and M phases, and it was mapped to an origin of DNA replication in asynchronous populations of cells using in vivo cross linking (Homesley et al., 2000). Finally, when yeast cells bearing a temperature sensitive allele of *MCM10* are shifted to the nonpermissive temperature after a G_1 arrest, the Mcm2-7 complex dissociates from chromatin while ORC is unaffected (Homesley et al., 2000). Together, these results have led to the model that Mcm10 is a component of the pre-RC that loads independently of ORC and that is required to mediate the association of Mcm2-7 with origins of replication.

To better understand the role of Mcm10 in DNA replication, we have cloned the *Xenopus* homolog of *MCM10* and characterized its function in *Xenopus* egg extracts. We find that immunodepletion of XMcm10 from NPE and EC results in a 5- to 10-fold decrease in DNA replication that can be rescued by the addition of recombinant XMcm10. XMcm10 does not bind chromatin in egg cytosol, but strongly associates with chromatin upon the addition of NPE in a process that requires the presence of the XMcm2-7 complex within pre-RCs and temporally precedes the chromatin loading of XCdc45 and XRPA. In extracts depleted of XMcm10, XMcm2-7 loading to form pre-RCs is unaffected while the recruitment of XCdc45 and XRPA to the chromatin is defective. Together our data argue that XMcm10 is an essential replication initiation factor that is not involved in pre-RC assembly but instead facilitates the loading of XCdc45 onto the chromatin.

Results

XMcm10 Is Enriched in Nuclear Extracts

A database search identified a *Xenopus* EST clone (db25e09) that was a potential homolog of *S. cerevisiae MCM10*. The 3.3 kb cDNA clone encoded a hypothetical open reading frame of 860 amino acids that was 10% identical and 21% homologous to *S. cerevisiae MCM10* and 56% identical and 70% homologous to human *MCM10*. This sequence is identical to the previously deposited Genbank sequence, AF314535. A fragment of the XMcm10 encoding amino acids 306–810 was purified as a His6 fusion protein and injected into rabbits. The resulting antiserum, but not the preimmune serum, specifically recognized a 100 kDa band in *Xenopus* egg cytosol (Figure 1A, lanes 1 and 2). We also compared the abundance of XMcm10 in egg cytosol and NPE, and found it to be approximately 10 times more concentrated in NPE than in egg cytosol (Figure 1B, compare lanes 1 and 4). This indicates that XMcm10 is efficiently imported into the nucleus in *Xenopus* egg extracts, in agreement with previous experiments in *S. cerevisiae*, *S. pombe*, and human cells (Izumi et al., 2000; Kawasaki et al., 2000; Merchant et al., 1997).

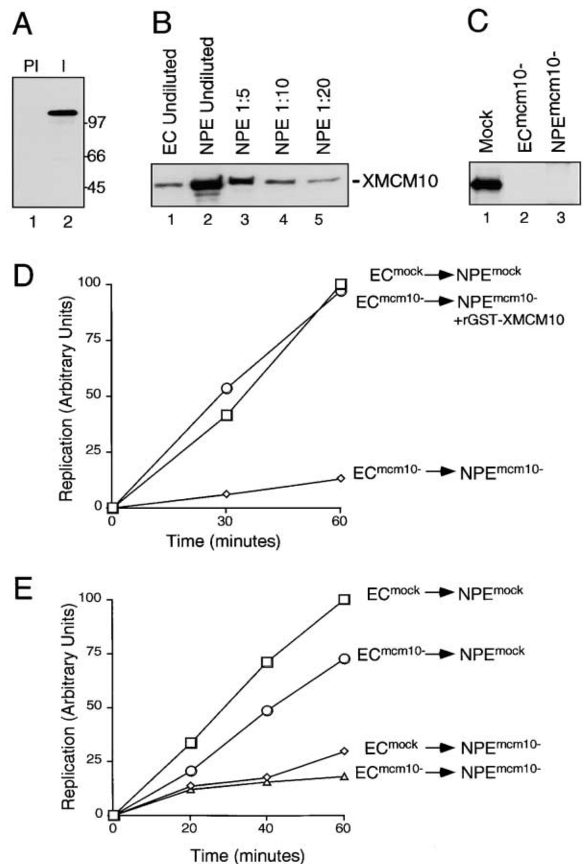

Figure 1. *Xenopus* Mcm10 Is a Nuclear Protein Required for Efficient DNA Replication

(A) Characterization of the XMcm10 antiserum. 0.5 μl of egg cytosol (EC) was immunoblotted with anti-XMcm10 antiserum (lane 2) or the corresponding preimmune serum (lane 1).

(B) XMcm10 is 10-fold enriched in NPE compared to EC. 0.5 μl EC (lane 1), 0.5 μl NPE (lane 2), 0.1 μl NPE (lane 3), 0.05 μl NPE (lane 4), and 0.025 μl NPE (lane 5) were immunoblotted with anti-XMcm10 antibody.

(C) Egg cytosol and NPE are efficiently depleted of XMcm10. 0.5 μl mock-depleted EC (lane 1), 0.5 μl XMcm10-depleted egg cytosol (lane 2), and NPE (lane 3) were immunoblotted with XMcm10 antibody.

(D) XMcm10 is essential for DNA replication. Sperm chromatin (10,000/μl final concentration) was incubated with XMcm10-depleted egg cytosol for 30 min, followed by XMcm10-depleted NPE (diamonds), or XMcm10-depleted NPE supplemented with 40 ng/μl final concentration GST-XMcm10 (circles). The relative amount of DNA replication was measured 30 and 60 min after NPE addition and compared with replication in mock-depleted extracts (squares).

(E) The XMcm10 in egg cytosol is dispensable for efficient DNA replication. Sperm chromatin was incubated with egg cytosol and replication was measured at various times following the addition of NPE. (Squares), mock-depleted EC, mock-depleted NPE; (circles) Mcm10-depleted EC, mock-depleted NPE; (diamonds), mock-depleted EC, Mcm10-depleted NPE; (triangles), Mcm10-depleted EC, Mcm10-depleted NPE.

XMcm10 Is Required for DNA Replication

To determine whether XMcm10 is required for DNA replication, we depleted it from *Xenopus* egg extracts. As shown in Figure 1C, anti-XMcm10 serum efficiently depleted XMcm10 protein from egg cytosol and NPE. Western blotting with serial dilutions of mock-depleted

extract showed that the depletion was greater than 99% (data not shown). Extracts depleted of XMcm10 exhibited a 8-fold decrease in DNA replication compared to mock-depleted extracts (Figure 1D, compare diamonds and squares), and this defect was completely reversed by addition of recombinant GST-XMcm10 (Figure 1D, circles). Therefore, the replication defect in XMcm10-depleted extracts was due to the selective removal of XMcm10. The addition of recombinant GST-XMcm10 to mock-depleted extracts had little effect on DNA replication (data not shown). These data show that XMcm10 is essential for DNA replication in *Xenopus* extracts.

We next examined whether XMcm10 must be present in egg cytosol, NPE, or both to support DNA replication (Figure 1E). Depletion of XMcm10 from the egg cytosol had only a modest effect on DNA replication (Figure 1E, circles), while depletion of XMcm10 from NPE alone led to a large decrease in DNA replication (Figure 1E, diamonds). Depletion of XMcm10 from both extracts abolished replication to a similar extent as depletion from NPE alone (Figure 1E, triangles). We showed previously that if XMcm2-7 complexes do not bind to chromatin in egg cytosol, they are unable to do so after the addition of NPE (Walter, 2000). Therefore, the lack of a requirement for XMcm10 in egg cytosol suggests that XMcm10 is not required for XMcm2-7 binding.

Kinetics of Chromatin Association of XMcm10

Since both human and budding yeast Mcm10 are tightly associated with chromatin (Homesley et al., 2000; Izumi et al., 2000; Kawasaki et al., 2000), we tested whether XMcm10 binds to chromatin during DNA replication. In contrast to XMcm7, very little XMcm10 was bound to sperm chromatin incubated in egg cytosol (Figure 2A, lane 2). However, a large increase in chromatin binding by XMcm10 occurred within 10 min after the addition of NPE (Figure 2A, lane 4). Over time, XMcm10 was displaced from the chromatin with identical kinetics as the XMcm2-7 complex (Figure 2A, lanes 4–6). When DNA replication was inhibited by the addition of the Cdk2 inhibitor $p27^{Kip}$ (as seen by the inhibition of XCdc45 and XRPA loading), XMcm10 associated with chromatin but did not dissociate over time (Figure 2A, lanes 7–9). Similarly, when DNA replication was inhibited by aphidicolin (as seen by the enhanced loading of XRPA [Walter, 2000]), XMcm10 bound to chromatin, but did not dissociate over time (Figure 2A, lanes 10–12). Thus, XMcm10 binds to chromatin before DNA replication is initiated, and it is displaced from the chromatin with the same kinetics as the XMcm2-7 complex as replication forks progress. The displacement of XMcm10 from chromatin during DNA replication is consistent with the finding in human cells that XMcm10 is more abundant on chromatin in S phase than in G_2 (Izumi et al., 2000).

That XMcm10 bound to chromatin in the presence of $p27^{Kip}$ (Figure 2A, lanes 7–9) suggested that XMcm10 might bind to chromatin before Cdc45, whose loading is dependent on Cdk activity. To test this, we performed a detailed time course of chromatin loading (Figure 2B). XMcm10 associated with chromatin less than 2 min after the addition of NPE (Figure 2B, lane 2), whereas chromatin association of XCdc45 and XRPA occurred ~6 min after the addition of NPE (Figure 2B, lane 4). Therefore,

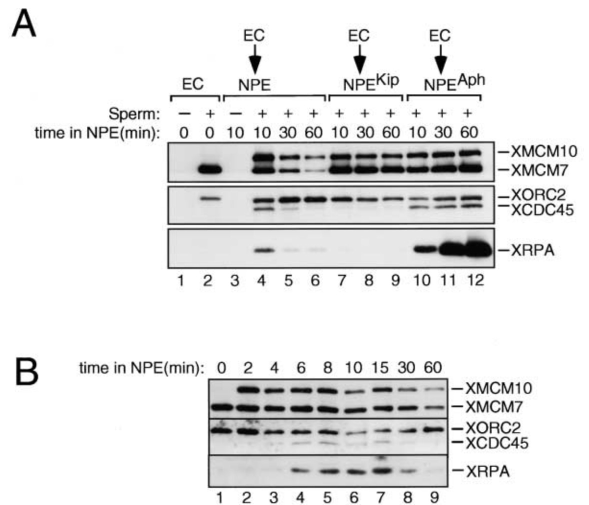

Figure 2. XMcm10 Associates with Chromatin after Pre-RC Assembly but before XCdc45 Loading

(A) Sperm chromatin was incubated with egg cytosol at 10,000/μl final concentration and isolated after 30 min (lane 2) or 10, 30, and 60 min following the addition of NPE (lanes 3–6), NPE supplemented with 1 μM GST-$p27^{Kip}$ (lanes 7–9), or NPE containing 50 μg/ml Aphidicolin (lanes 10–12). The reactions shown in lanes 1 and 3 lacked sperm chromatin. Purified chromatin was immunoblotted for XMcm10, XMcm7, Xorc2, XCdc45, and XRpa34, as indicated.

(B) Sperm chromatin was incubated with egg cytosol, and the association of XMcm10, XMcm7, Xorc2, XCdc45, and XRpa34 determined at various times after the addition of NPE.

XMcm10 normally associates with chromatin before XCdc45 and XRPA.

Chromatin Association of XMcm10 Requires a Functional Pre-RC but Is Independent of Cdc7 Activity

Given the genetic and physical interaction of yeast Mcm10 with other members of the yeast Mcm2-7 complex, we tested whether the XMcm2-7 complex is required for the chromatin loading of XMcm10. We used antibodies against XMcm7 to deplete the XMcm2-7 complex from egg cytosol and then examined the chromatin association of XMcm10 after addition of NPE. It was not necessary to deplete XMcm7 from NPE, because de novo binding of XMcm2-7 does not occur after addition of NPE (Walter et al., 1998; Walter, 2000). XMcm2-7-depleted extracts were unable to recruit XMcm10 to the chromatin (Figure 3A, compare lanes 3 and 4). Similarly, the addition to egg cytosol of geminin, which blocks XMcm2-7 loading through its association with Cdt1 (Tada et al., 2001; Wohlschlegel et al., 2000), also prevented the recruitment of XMcm10 to replication origins (Figure 3B). Together, these data suggest that the presence on chromatin of the XMcm2-7 complex is required for the recruitment of XMcm10 to replication origins.

We next determined whether XCdc7 was required for chromatin binding of XMcm10. We find that XMcm10 was efficiently loaded onto chromatin in the absence of XCdc7 (Figure 3C, lane 4). The effectiveness of the XCdc7-depletion was demonstrated by the absence of XRPA chromatin binding in the depleted extracts (Figure

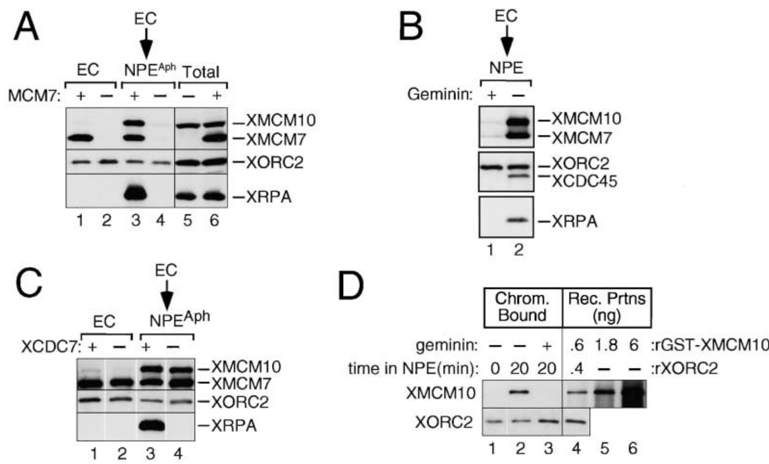

Figure 3. The Association of XMcm10 with Chromatin Requires XMcm2-7 but Is Independent of XCdc7

(A) XMcm10 does not bind to chromatin in XMcm7-depleted extracts. Sperm chromatin was incubated with mock-depleted (lanes 1 and 3) or XMcm7-depleted egg cytosol (lanes 2 and 4). In lanes 1 and 2, chromatin was isolated after incubation in egg cytosol, whereas in lanes 3 and 4, it was isolated after a further 20 min incubation in NPE containing aphidicolin. Chromatin and 0.5 μl aliquots of mock-depleted (lane 6) and XMcm7-depleted egg cytosol (lane 5) were immunoblotted for XMcm10, XMcm7, XOrc2, and XRpa34, as indicated.

(B) Geminin inhibits chromatin binding of XMcm10. Sperm chromatin was incubated with egg cytosol containing 150 nM human geminin (lane 1) or buffer (lane 2), and isolated after a further 10 min incubation with NPE.

(C) Chromatin association of XMcm10 does not require XCdc7. Sperm chromatin was incubated with mock-depleted (lanes 1 and 3) or XCdc7-depleted egg cytosol (lanes 2 and 4). After 30 min, chromatin was isolated (lanes 1 and 2) or incubated for a further 30 min with mock-depleted or XCdc7-depleted NPE containing aphidicolin and then isolated (lanes 3 and 4).

(D) The ratio of XMcm10 to XOrc2 on chromatin is ~2:1. Sperm chromatin was isolated after incubation in egg cytosol (lane 1), egg cytosol followed by a 20 min incubation in NPE containing aphidicolin (lane 2), or egg cytosol containing 150 nM geminin followed by a 20 min incubation in NPE containing aphidicolin (lane 3). Chromatin-bound proteins from 10,000 sperm were immunoblotted alongside known quantities of purified GST-XMcm10 or his-XOrc2 (lanes 4–6). Lane 4 contains equimolar amounts of XMcm10 and XOrc2.

3C, lane 4). Figure 2A showed that the addition of the Cdk2 inhibitor p27^{Kip} did not inhibit chromatin binding by XMcm10. Therefore, recruitment of XMcm10 to replication origins is independent of both Cdk2 and Cdc7, but requires the XMcm2-7 complex.

It was important to determine how much XMcm10 loads onto origins at the onset of DNA replication. To this end, sperm chromatin was incubated in egg cytosol followed by NPE containing aphidicolin. Chromatin was then isolated and analyzed on a Western blot alongside known quantities of purified GST-XMcm10. We found that ~1 ng of XMcm10 is loaded onto 10,000 sperm (Figure 3D, compare lane 2 with lanes 4 and 5). Since each sperm contains 2.9×10^9 basepairs of DNA, XMcm10 binds on average once every 5000 basepairs. In the same experiment, ~0.3 ng XOrc2 was found to bind to 10,000 sperm, or one molecule per 11,000 basepairs (Figure 3D, compare lanes 1 and 4), which is similar to previous reports (Rowles et al., 1996; Walter and Newport, 1997) and close to the experimentally determined replicon size of ~10 kb (Blow et al., 2001; Hyrien and Mechali, 1993; Mahbubani et al., 1992; Walter and Newport, 1997). Given that all the XMcm10 loaded in this experiment was geminin sensitive (Figure 3D, lane 3), we conclude that roughly two molecules of XMcm10 bind per origin of DNA replication at the onset of DNA replication. The concentration of XMcm10 in *Xenopus* egg cytosol is 16 ng/μl (data not shown).

XMcm10 Is Required for Chromatin Binding of XCdc45 and Origin Unwinding, but Not for XMcm2-7 Complex Recruitment

Experiments in yeast strongly suggested that Mcm10 is required for the initiation of DNA replication (Merchant et al., 1997). To determine at what step of replication initiation XMcm10 performs its function, we carried out chromatin binding experiments in XMcm10-depleted extracts (Figure 4A). Sperm chromatin was incubated in XMcm10-depleted egg cytosol, followed by the addition of XMcm10-depleted NPE containing aphidicolin. The aphidicolin served to arrest the system after initiation had occurred. Under these conditions, normal levels of XMcm7 bound to chromatin (Figure 4A, compare lanes 1 and 2). Sperm isolated from XMcm10-depleted egg cytosol before addition of NPE also contained the same amount of XMcm7 and XMcm3 as mock-depleted extracts (data not shown). Strikingly, in the absence of XMcm10, XCdc45 and XRPA binding in NPE was severely reduced (Figure 4A, compare lanes 1 and 2), and the reduced binding was reversed by the addition of recombinant XMcm10 (Figure 4A, lane 3). In this experiment, depletion of XMcm10 caused an ~6-fold reduction in DNA replication that was fully rescued by GST-XMcm10, and the overall efficiency of DNA replication was ~100% (Figure 4B, see legend). Similar defects in XRPA and XCdc45 binding were observed in extracts lacking aphidicolin (data not shown). We also examined the binding of XCdc7 to chromatin that normally occurs after NPE addition (Walter, 2000) in XMcm10-depleted extracts. We found a small but reproducible decrease in the amount of chromatin-bound XCdc7 in XMcm10-depleted extracts (Figure 4C). Thus, although our data show that XCdc7 loads onto chromatin independently of XMcm10, we cannot rule out the possibility that there is a pool of XCdc7 whose chromatin association is XMcm10 dependent. Together, these experiments show that XMcm10 is not required for prereplicative complex assembly, but rather for the recruitment of XCdc45 and RPA to origins of replication.

The lack of XCdc45 and XRPA binding in XMcm10-depleted extracts suggests that XMcm10 is required for origin unwinding (Walter and Newport, 2000). To test this directly, a circular plasmid was incubated in egg cytosol followed by NPE containing aphidicolin, and its

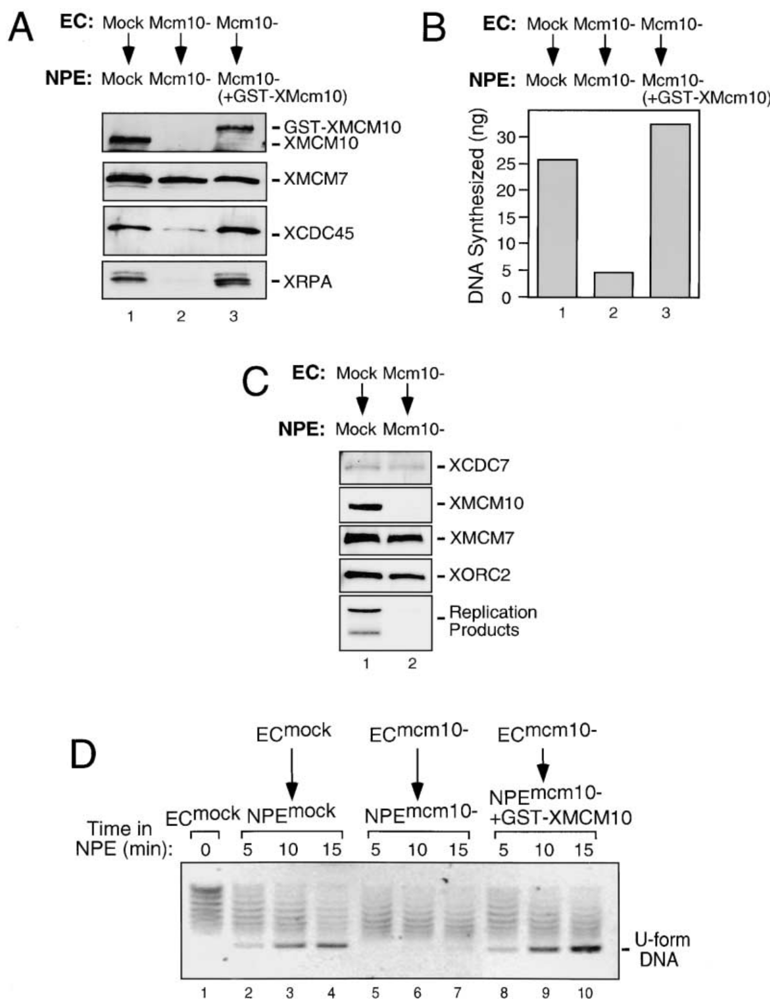

Figure 4. XMcm10 Is Required for the Chromatin Association of XCdc45 and Origin Unwinding

(A) Sperm chromatin was incubated with mock-depleted egg cytosol followed by mock-depleted NPE (lane 1) or XMcm10-depleted egg cytosol followed by XMcm10-depleted NPE (lanes 2 and 3). In lane 3, the NPE contained 40 ng/μl GST-XMcm10, and NPE in all three reactions was supplemented with 50 ng/μl human cyclin A/Cdk2 (see Experimental Procedures) and aphidicolin. Chromatin association of XMcm7, XCdc45, and XRPA was determined by immunoblotting after 30 min incubation in NPE.

(B) The same reactions as in (A), but lacking aphidicolin, were supplemented with [α-^{32}P]dATP, and replication from 8,300 sperm was measured 30 min after NPE addition. Because the amount of input DNA (27 ng) matched the amount of newly synthesized DNA (see Figure 4B), replication efficiency was ~100%.

(C) Sperm chromatin was incubated with mock-depleted egg cytosol followed by mock-depleted NPE (lane 1) or XMcm10-depleted egg cytosol followed by XMcm10-depleted NPE (lane 2). Both mock and XMcm10-depleted NPE were treated with p27^{KIP} to arrest replication initiation after XCdc7 loading (Walter, 2000). Chromatin association of XMcm7, XOrc2, XCdc7, and XMcm10 was determined by immunoblotting after 20 min incubation in NPE. DNA replication was also assessed in mock and XMcm10-depleted extracts after 30 min incubation in NPE.

(D) Origin unwinding does not occur in XMcm10-depleted extracts. pBS (40 ng/μl final concentration) was incubated with mock-depleted (lanes 1–4) or Mcm10-depleted egg cytosol for 30 min (lanes 5–10). DNA was then isolated (lane 1) or further incubated after addition of mock-depleted NPE (lanes 2–4), XMcm10-depleted NPE (lanes 5–7), or XMcm10-depleted NPE containing 40 ng/μl GST-XMcm10 (lanes 8–10), and then isolated. Origin unwinding was monitored by the appearance of negatively supercoiled "U" form DNA on a chloroquine agarose gel.

topology was analyzed on an agarose gel containing chloroquine. Origin unwinding occurs after NPE addition and can be separated into two steps (Walter and Newport, 2000). The first step requires Mcm2-7, Cdk2, Cdc7, and Cdc45 but is RPA-independent and involves a significant but limited supercoiling of the plasmid (see Figures 3A–3F in Walter and Newport, 2000). The second step requires a single-stranded DNA binding protein (RPA or *E. Coli* SSB) and results in a highly unwound species (U form DNA). The amount of U form DNA generated in XMcm10-depleted extracts was severely reduced compared to mock-depleted extracts (Figure 4D, lanes 2–4 versus 5–7), and this defect was rescued by the addition of recombinant XMcm10 (Figure 4D, lane 8–10). Moreover, in the absence of XMcm10, there was no partial supercoiling of the plasmid. The small change in linking number that is observed upon addition of NPE (Figure 4D, compare lanes 1 and 5) is ORC-independent (Walter et al., 1998) and therefore not related to initiation of DNA replication. Therefore, using the DNA topology assay, we find that origin unwinding is blocked at an early step in XMcm10-depleted extracts. The results of the chromatin binding and plasmid supercoiling assays therefore both show a requirement for XMcm10 at an early stage of origin unwinding.

Discussion

In this paper, we report the cloning and characterization of the *Xenopus* homolog of the replication factor *MCM10*. Using the *Xenopus* cell-free DNA replication system, we show that XMcm10 is required for DNA replication and that it is recruited to origins before XCdc45 and XRPA. XMcm10 recruitment is dependent on the XMcm2-7 complex, but it is independent of Cdk2 and Cdc7 activities. We also show that XMcm10 is required for the recruitment of XCdc45 and XRPA to replication origins, and as such, its depletion from extracts leads to a defect in origin unwinding. Based on these findings, we present a model for XMcm10 function in which XMcm10 is not required for pre-RC assembly but instead participates in the activation of pre-RCs at the onset of S phase to facilitate their conversion to active replication forks (Figure 5).

MCM10 was initially identified in yeast as a gene re-

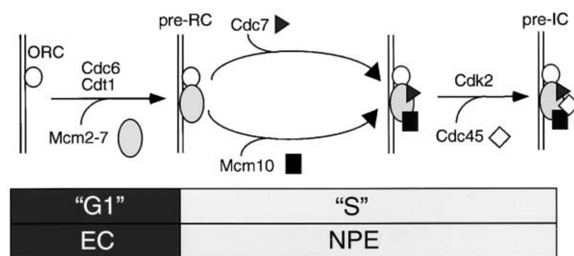

Figure 5. Model for the Assembly of Replication Factors at Origins of Replication in *Xenopus* Extracts

quired for chromosomal DNA replication and plasmid stability (Merchant et al., 1997; Solomon et al., 1992). Biochemical and genetic studies have since given rise to the current model for Mcm10 function in which Mcm10 is a critical component of the pre-RC that cooperates with ORC to recruit the Mcm2-7 complex to replication origins (Homesley et al., 2000; Izumi et al., 2000; Kawasaki et al., 2000; Merchant et al., 1997). In contrast, we have provided three lines of evidence that the *Xenopus* homolog of Mcm10 is not required for the recruitment or the maintenance of the XMcm2-7 complex on chromatin. First, DNA replication in *Xenopus* extracts does not requires XMcm10 to be present in egg cytosol during the formation of pre-RCs (Figure 1E). Importantly, this incubation in egg cytosol represents the only window of opportunity for pre-RC assembly in this system. Second, we show that virtually no XMcm10 associates with chromatin in egg cytosol during pre-RC assembly, whereas one to two XMcm10 molecules bind to origins upon the addition of NPE (Figures 2A and 3D). Finally, in XMcm10-depleted extracts, there is no reduction in the efficiency of XMcm2-7 loading onto chromatin (Figure 4A). Differences in our studies and the yeast studies reporting the requirement of Mcm10 for the stable association of Mcm2-7 with chromatin may result from the fact that those studies assayed the presence of Mcm2-7 in the general chromatin fraction and not just at origins. Alternatively, discrepancies with the yeast data may reflect genuine differences between *X. laevis* and *S. cerevisiae* with regard to the order of events during replication initiation.

Although XMcm10 is not required for the stable chromatin loading of the Mcm2-7 complex, we find that the converse is true. The recruitment of XMcm10 to origins requires the prior chromatin association of the XMcm2-7 complex (Figures 3A and 3B). This finding is consistent with the strong genetic and physical interactions between Mcm10 and the Mcm2-7 complex (Homesley et al., 2000; Merchant et al., 1997) and also provides the molecular basis for the localization of XMcm10 to replication origins. These observations raise the question whether XMcm10 should be considered a component of the prereplication complex, which was originally defined as the protein complex that assembles at origins of DNA replication in the G_1 phase of the cell cycle in budding yeast (Diffley et al., 1994). Since egg cytosol and NPE recapitulate key features of the G_1 and S phases of the cell cycle, respectively (see Introduction), and because XMcm10 loads onto origins only after NPE addition, we suggest that chromatin binding of XMcm10 is an S phase event. This interpretation is supported by the recent observation by Izumi and colleagues that the human Mcm10 binds to chromatin at the onset of S phase in HeLa cells (Izumi et al., 2001). It still remains unclear what triggers Mcm10 binding to chromatin. Our data suggest that Cdk activity is not required for this event. Therefore, it may be that Mcm10 binds to pre-RCs when a sufficient concentration of this protein is present in cells. This model is consistent with our finding that Mcm10 binds to chromatin only in NPE, where it is enriched, and that it binds soon after its expression level increases in Hela cells (Izumi et al., 2001).

At the onset of S phase, pre-RCs are converted into initiation complexes by the Cdk- and Cdc7-dependent chromatin loading of Cdc45 onto replication origins (Jares and Blow, 2000; Takisawa et al., 2000; Walter, 2000; Zou and Stillman, 2000). Our results show that the recruitment of XCdc45 and XRPA to replication origins also requires XMcm10 (Figure 4A). The involvement of Mcm10 in this process provides an explanation for the synthetic lethality previously seen between mutant alleles of *CDC45* and *MCM10* (Homesley et al., 2000). Interestingly, the two mutant alleles of the Mcm2-7 complex that were shown to specifically suppress the *mcm10-1* mutant were initially identified as suppressors of *cdc45-1*, further emphasizing the functional relationship between Cdc45 and Mcm10 (Homesley et al., 2000). Thus, our findings that XMcm10 is involved in the recruitment of XCdc45 to replication origins in an XMcm2-7-dependent fashion is consistent with previous genetic studies. Although the exact mechanism by which Mcm10 functions to recruit Cdc45 is unclear, it is intriguing that Mcm10 is recruited to replication origins independently of both Cdc7 and Cdk2 activity, yet all three are required for Cdc45 loading. It is tempting to speculate that the phosphorylation of Mcm10 by Cdc7 and/or Cdk2 may be a critical step in the recruitment of Cdc45 to origins of replication.

Our results suggest that the chromatin association of Mcm10 is the earliest detectable step in the activation of pre-RCs at the onset of DNA replication, as it occurs before Cdc45 loading and independently of Cdc7 and Cdk2. This finding raises the possibility that Mcm10 may play an important role in determining which origins fire and the time in S phase at which they fire. Previous studies in budding yeast have identified the chromatin association of Cdc45 as an important marker for the timing of origin activation and also suggested that the S phase checkpoint may regulate the chromatin association of Cdc45 through the action of the checkpoint kinase Rad53 (Aparicio et al., 1999; Costanzo et al., 2000; Zou and Stillman, 2000). Our data raise the possibility that the regulated chromatin association of Cdc45 seen in those studies could also be affected by the regulated chromatin association of Mcm10. Evidence for involvement of Mcm10 in the DNA replication checkpoint comes from two different observations. One is that *MCM10* mutants are synthetically lethal with *RAD53* mutants (Kawasaki et al., 2000). Second, inactivation of Mcm10 in yeast cells during S phase leads to a loss of chromosome integrity that could result from the lack of an intact S phase checkpoint (Kawasaki et al., 2000).

Although our results clearly indicate a role for Mcm10

in initiation complex formation, they do not rule out an additional role for Mcm10 in elongation. Our finding that XMcm10 is displaced from the chromatin with identical kinetics to the XMcm2-7 complex is suggestive of a role in elongation. A recent study in *S. cerevisiae* showed that Mcm10 is required for the completion of S phase after release from a hydroxyurea block (Kawasaki et al., 2000). Since no new initiation events are required to complete S phase under these conditions, these results suggest that Mcm10 is also required for the elongation stage of DNA replication. The genetic interactions of *MCM10* with subunits of Polϵ, Polδ, and DNA2, proteins known to be involved in elongation, also support a role for Mcm10 in elongation (Kawasaki et al., 2000; Liu et al., 2000). Could the role of Mcm10 in elongation be similar to its role in initiation? Studies of a *cdc45* degron mutant showed that newly synthesized Cdc45 could be functionally reincorporated into stalled replication forks during hydroxyurea arrest and then subsequently perform its essential role in elongation (Tercero et al., 2000). As this recruitment of Cdc45 to replication forks is thought to be independent of pre-RCs, it is tempting to speculate that this recruitment of Cdc45 to stalled replication forks may require Mcm10. Thus, it may be possible that Mcm10 is not only required for the recruitment of Cdc45 to the replication origins during the initiation of DNA replication but also cooperates with Cdc45 during elongation and is required for its stable association with replication forks.

Experimental Procedures

Cloning of XMcm10 and Plasmid Construction

The cDNA encoding XMcm10 (dbEST Id: 4548867) was identified by searching the *Xenopus* EST database for genes with homology to *S. cerevisiae MCM10* and obtained from the Washington University Genome Sequencing Center. We have sequenced the entire cDNA and find that it is identical to a previsouly deposited sequence in Genbank (AF314535). The entire open reading encoding amino acids 1–860 was PCR amplified using primers containing a BglII site in the 5′ primer and a SalI site in the 3′ primer. The PCR product was digested with BglII and SalI and ligated to a variant of pGEX-2T digested with BamHI and XhoI. We also cloned a fragment of XMcm10 encoding amino acids 306–810 from IMAGE clone PBX0047E07 into pET28a for expression as a His-tagged fusion protein. Briefly, the plasmid was digested with BamHI and NotI, and the resulting 1.6 kb fragment was ligated to pET28a (Novagen, Madison, WI) that had been digested with the same enzymes.

Production of Antibodies against XMcm10

pET28a-XMcm10 (amino acids 306–810) was transformed into bacterial strain BL21(DE3), and expression of the His-tagged fusion protein was induced by the addition of IPTG to a final concentration of 1 mM. The protein was then solubilized in 8 M urea and purified over a Nickel-NTA agarose column as described by the manufacturer (Qiagen, Valencia, CA). Rabbits were immunized with this antigen (Cocalico Biologicals, Reamstown, PA), and the resulting antiserum reacted specifically with His6-XMcm10 (amino acids 306–810).

Purification of GST-Mcm10

pGEX-XMcm10 was expressed in BL21(DE3) cells and purified using GSH-agarose according to the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). The purified protein was then dialyzed against egg lysis buffer (ELB; 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM HEPES [pH 7.7]), and its concentration was estimated using the Biorad protein assay.

Immunological Methods

XMcm10 was depleted from 1 volume of egg cytosol or NPE by three sequential incubations of 2, 2, and 12 hr with 0.2 volumes of protein A Sepharose fast flow (Pharmacia) that had been prebound to 0.6 volumes of XMcm10 antiserum or the corresponding preimmune serum. Depletions of XCdc7 and XMcm7 were described previously (Walter, 2000). Western blotting with XMcm10 antiserum was performed by using antiserum diluted to 1:5000. Western blotting with antibodies against XMcm7, XCdc7, XOrc2, XCdc45, and XRPA were described previously (Walter and Newport, 2000; Walter et al., 1998; Walter, 2000).

DNA Replication, DNA Unwinding, and Chromatin Binding Assays

Extract preparation and replication assays were carried out as described (Walter et al., 1998). Chromatin binding assays were performed as described in Walter (2000). DNA unwinding assays were performed according to Walter and Newport (2000). The lengthy immunodepletion procedure required to remove XMcm10 (see above) often led to significant nonspecific inactivation of NPE through loss of Cdk activity. Therefore, in the experiments presented in Figures 4A and 4B, NPE was supplemented with 50 ng/μl final concentration cyclin A/Cdk2 (Wohlschlegel et al., 2001). The amount of DNA synthesized in Figure 4B was calculated as described (Blow and Laskey, 1986) assuming 50 μM endogenous pools of dNTPs.

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