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# Localization of MCM2-7, Cdc45, and GINS to the Site of DNA Unwinding during Eukaryotic DNA Replication

Short Article

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## Summary

**Little is known about the architecture and biochemical composition of the eukaryotic DNA replication fork. To study this problem, we used biotin-streptavidin-modified plasmids to induce sequence-specific replication fork pausing in *Xenopus* egg extracts. Chromatin immunoprecipitation was employed to identify factors associated with the paused fork. This approach identifies DNA pol α, DNA pol δ, DNA pol ε, MCM2-7, Cdc45, GINS, and Mcm10 as components of the vertebrate replisome. In the presence of the DNA polymerase inhibitor aphidicolin, which causes uncoupling of a highly processive DNA helicase from the stalled replisome, only Cdc45, GINS, and MCM2-7 are enriched at the pause site. The data suggest the existence of a large molecular machine, the "unwindosome," which separates DNA strands at the replication fork and contains Cdc45, GINS, and the MCM2-7 holocomplex.**

## Introduction

Eukaryotic DNA replication takes place in three stages, prereplication complex (pre-RC) assembly, initiation, and elongation (Blow and Dutta, 2005). Pre-RC assembly occurs during G1 and involves ORC-, Cdc6-, and Cdt1-dependent recruitment of the MCM2-7 complex to origins. At the G1/S transition, Mcm10, Cdc45, Dpb11, GINS, and Sld2 are recruited to the origin. Together with DDK and CDK protein kinases, these factors activate a chromatin-associated "replicative DNA helicase." This helicase unwinds the origin, and DNA pol α, DNA pol δ, and DNA pol ε are recruited to form the intact replisome. During elongation, a helicase unwinds DNA at the replication fork, and leading and lagging strands are synthesized in a coordinated fashion.

The MCM2-7 complex, a ring-shaped, heterohexameric AAA+ ATPase, is the best candidate for the replicative DNA helicase (reviewed in Takahashi et al. [2005]). In yeast, at least five out of six MCM2-7 subunits are required for replication fork progression (Labib et al., 2000), and chromatin immunoprecipitation (ChIP) experiments indicate that Mcm4 and Mcm7 travel with the DNA replication fork (Aparicio et al., 1997). In *Xenopus* egg extracts, targeting the MCM2-7 complex with antibodies or with retinoblastoma protein inhibits a replisome-associated DNA helicase (Pacek and Walter, 2004; Shechter et al., 2004). Finally, a purified complex consisting of Mcm4, Mcm6, and Mcm7 exhibits DNA helicase activity (Ishimi et al., 1998). Despite this evidence in favor of MCM2-7 being the replicative helicase, it remains puzzling that purified MCM2-7 is devoid of helicase activity and that immunofluorescence studies fail to show colocalization of Mcm proteins with replication factories in vertebrate cells (reviewed in Takahashi et al. [2005]).

Besides MCM2-7, several other factors play essential roles in origin unwinding and elongation and therefore may function as replicative helicase cofactors. For example, Cdc45 is an essential component of the replication fork (Aparicio et al., 1997; Pacek and Walter, 2004; Tercero et al., 2000), and antibodies against Cdc45 block DNA unwinding during replication elongation in *Xenopus* egg extracts (Pacek and Walter, 2004). Moreover, Cdc45 can be coimmunoprecipitated with MCM proteins as well as DNA helicase activity from a chromatin fraction (Kubota et al., 2003; Masuda et al., 2003; Zou and Stillman, 1998). GINS and Mcm10 are also required for Cdc45 loading, origin unwinding, and progression of replication forks (Kanemaki et al., 2003; Kubota et al., 2003; Ricke and Bielinsky, 2004; Takayama et al., 2003; Wohlschlegel et al., 2002). Although it has been proposed that Mcm10 and GINS function in concert with DNA polymerase α and ε, respectively, the data are also consistent with these proteins functioning as helicase cofactors. In this paper, we describe an approach to identify replisome-associated factors in a vertebrate model system and, further, to discern which of these are specifically associated with the replicative DNA helicase.

## Results and Discussion

Due to the lack of highly active and sequence-specific origins of DNA replication, it has been impossible to study replication elongation in vertebrates using ChIP. To overcome these difficulties, we have exploited a soluble cell-free replication system derived from *Xenopus* eggs (Walter et al., 1998). In this system, plasmid DNA is first added to a *h*igh-speed *s*upernatant (HSS) of egg cytoplasm, which supports pre-RC assembly. Subsequent addition of a concentrated *n*ucleo*p*lasmic *e*xtract (NPE) stimulates replication initiation. Although initiation is sequence independent in this system, we reasoned that the replisome could be studied by ChIP if replication forks were forced to pause at a specific location on a plasmid (Figure 1A, left arrow).

It has been shown that a biotin-streptavidin (SA) complex blocks DNA unwinding by the purified MCM4-6-7 helicase in vitro (Kaplan et al., 2003; Shin et al., 2003).

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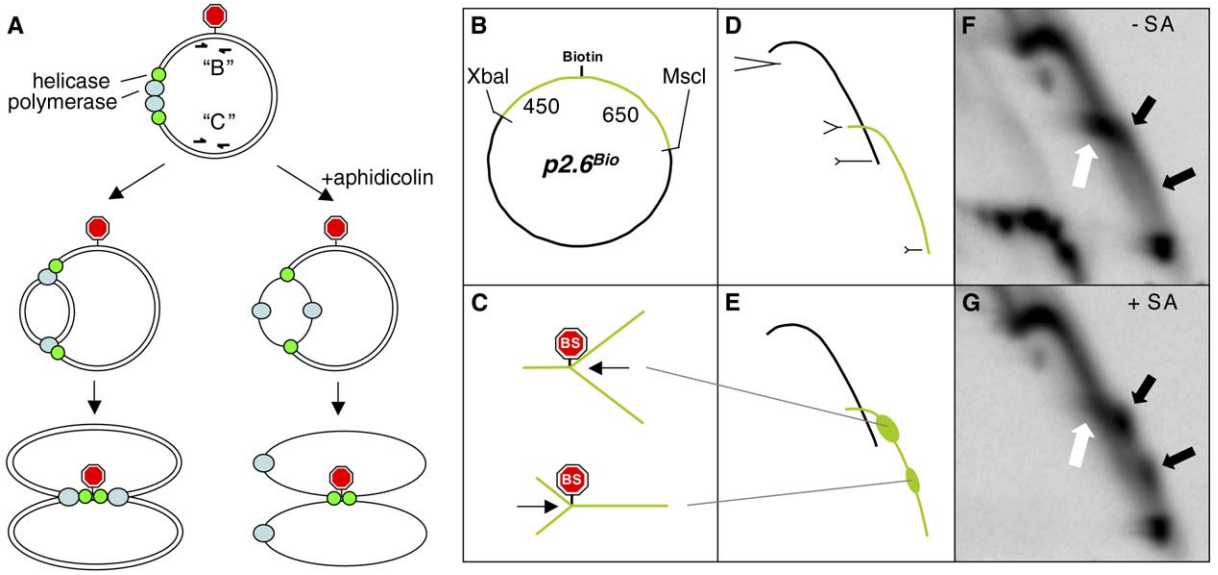


Figure 1. Biotin-SA Complexes Induce Replication Fork Pausing in *Xenopus* Egg Extracts

(A) Replication initiation leads to the assembly of two replisomes (helicase + polymerase) that move in opposite directions (left arrow). Given a replication fork barrier (red stop sign), replisomes accumulate at this site. In the presence of aphidicolin (right arrow), the helicase unwinds DNA in the absence of DNA synthesis and thus may become enriched at the replication fork barrier in the absence of other replisome components.

(B) Map of $p2.6^{Bio}$, including location of biotin and its distance in bp from XbaI and MscI sites.

(C) Expected structures generated when replication forks enter the 1.1 kb restriction fragment of $p2.6^{Bio}$ from the right (top) or left (bottom) and pause at the biotin-SA complex ("BS").

(D and E) Cartoon of expected Y arcs generated in 2D gels of $p2.6^{Bio}$ in the absence (D) and presence (E) of SA. Y structures corresponding to the ends of the arcs are indicated in (D).

(F and G) $p2.6^{Bio}$ was incubated with buffer (F) or SA (G) prior to replication. DNA was isolated 10 min after NPE addition and subjected to 2D gel analysis. Black arrows indicate sites of SA-induced replication fork pausing, and white arrows indicate the end of the Y arc derived from the 1.1 kb restriction fragment.

We therefore reasoned that biotin-SA might also impede movement of the endogenous replication fork. To test this, we generated $p2.6^{Bio}$, a 2.6 kb plasmid biotinylated at a single site (Figure 1B). To determine whether SA induces fork pausing when bound to $p2.6^{Bio}$, we used neutral-neutral 2D gel electrophoresis (Brewer and Fangman, 1987). $p2.6^{Bio}$ was mixed with buffer or streptavidin and then incubated with HSS, followed by addition of NPE containing radioactive dATP to visualize all newly replicated DNA. Ten minutes after NPE addition, DNA was isolated and digested with XbaI and MscI, yielding 1.5 and 1.1 kb fragments (Figure 1B, green and black segments) that should give rise to distinct "Y arcs" on 2D gels (Figure 1D). Indeed, the expected Y arcs were observed whether or not SA was present (Figures 1F and 1G). However, in the presence of SA, two distinct spots were visible on the Y arc derived from the 1.1 kb fragment (Figure 1G, black arrows). This observation is consistent with forks entering the restriction fragment from either direction, pausing at the asymmetrically positioned SA, and creating two distinct forked structures (Figure 1C). Importantly, much less radioactivity was observed at the top end of the small Y arc in the presence of SA than in its absence (compare white arrows in Figures 1F and 1G). Since the end of the Y arc corresponds to DNA restriction fragments that have replicated nearly to completion (see Figure 1D), their low abundance in the presence of SA suggests that replication fork pausing was effective. Therefore, biotin-SA complexes appear to cause replication fork pausing in *Xenopus* egg extracts.

We next examined the effects of SA on the efficiency of $p2.6^{Bio}$ replication. SA had no effect on the rate of radioactive dATP incorporation (see Figure S1A in the Supplemental Data available with this article online) or the decatenation of daughter molecules, as seen by the accumulation of monomeric daughter plasmids (Figure S1B, lanes 5 and 10). The absence of any effects on replication kinetics was not due to removal of the biotin (Figure S1C) and therefore is likely explained by one of three models. First, the helicase is eventually able to bypass the biotin-SA complex, as seen previously for an archaeal MCM complex (Shin et al., 2003). Second, even though one replication fork is blocked, the other completes synthesis of the plasmid substrate, consistent with the observed replisome independence in *E. coli* (Breier et al., 2005). Third, although plasmids smaller than 10 kb normally initiate once (Lucas et al., 2000), fork pausing may activate latent origins (Edwards et al., 2002). It is important to point out that, while individual 3 kb plasmids normally replicate in ~3 min, there is considerable asynchrony during initiation (Walter and Newport, 2000), which would explain why significant pausing does not necessarily slow down the overall kinetics of DNA synthesis. Thus, although SA does not affect the overall kinetics of DNA replication, the 2D gels clearly suggest that the replisome pauses at the biotin-SA locus.

We next used ChIP to examine the association of the MCM2-7 complex with the paused replisome. p2.6$^{Bio}$ was replicated in the presence and absence of SA. In mid S phase, chromatin was crosslinked with formaldehyde, sonicated, and precipitated with Mcm7 antibody, and the recovered DNA was amplified with two PCR primer pairs. Primer pair "B" amplifies the Biotin locus, and primer pair "C" amplifies a locus opposite the biotin (Figure 1A, top). We calculated the relative IP efficiency of the B and C loci using quantitative real-time PCR. Figure 2A shows that, in the presence of SA, the B locus was recovered much more efficiently than the C locus (compare columns 1 and 2), and the calculated ratio of B to C was 9.5. In the absence of SA, the B to C ratio was unity (Figure 2A, compare columns 3 and 4). These data suggest that Mcm7 is associated with the paused replisome.

We previously showed that the DNA polymerase inhibitor aphidicolin causes massive DNA unwinding ("hyperunwinding") in the absence of detectable DNA synthesis (Walter and Newport, 2000), indicating that the replicative DNA helicase can become uncoupled from the site of DNA synthesis. We speculated that, under these conditions, the uncoupled helicase might pause at the biotin-SA complex, allowing us to study its composition by ChIP (Figure 1A, right arrow). Strikingly, in the presence of aphidicolin, Mcm7 was also highly enriched at the biotin locus in a SA-dependent manner (Figure 2A, compare columns 5 and 6 with 7 and 8).

A potential complication arises in the analysis of MCM2-7 by ChIP. We previously showed that a large excess of "latent" MCM2-7 complexes is widely deposited on chromatin prior to replication initiation (Edwards et al., 2002). On each replicon, only a few of the bound MCM2-7 complexes are utilized for DNA replication (Edwards et al., 2002; Mahbubani et al., 1997), and the remaining, latent MCM complexes remain chromatin bound until they are displaced by passage of the replication fork (Madine et al., 1995). Thus, if the helicase stops short of the biotin, the remaining duplex DNA would likely contain residual latent MCM2-7 complexes that would cause enrichment of the biotin locus in Mcm7 ChIP. To confirm that the 3 kb plasmid was completely unwound, we examined histone H3 by ChIP. Before initiation ("early"), the B and C loci were both recovered efficiently (Figure S2A, columns 1 and 2). This result is expected, since egg extracts assemble any DNA into nucleosomes (Laskey et al., 1977). However, after replication initiated in the presence of aphidicolin ("late"), the recovery of B and C in H3 immunoprecitates was much lower, as expected if the entire plasmid was rendered single stranded (Figure S2A, columns 5 and 6). In contrast to histone H3, Mcm7 still bound to the biotin locus after initiation (Figure S2A, column 7). We conclude that the Mcm7 signal detected at the biotin locus is not due to latent MCM2-7 complexes but rather to Mcm7 that traveled to the site as part of the helicase complex.

The MCM complex can be separated into stable subassemblies containing Mcm4-6-7, Mcm2, or Mcm3/5 (Prokhorova and Blow, 2000). To examine members of all three subassemblies by ChIP, we also used Mcm2 and Mcm5 antibodies. These proteins were enriched at the biotin-SA complex in the absence of aphidicolin, indicating they are components of the replisome (Figures 2B and 2C, compare columns 1 and 2). Strikingly, Mcm2 and Mcm5 were also highly enriched at the biotin-SA locus in the presence of aphidicolin (Figures 2B and 2C, compare columns 5 and 6). As for Mcm7, Mcm2 and Mcm5 were detected at the biotin locus even after histone H3 was lost from this site (Figures S2B and S2C, compare columns 5 and 7). We conclude that Mcm2,

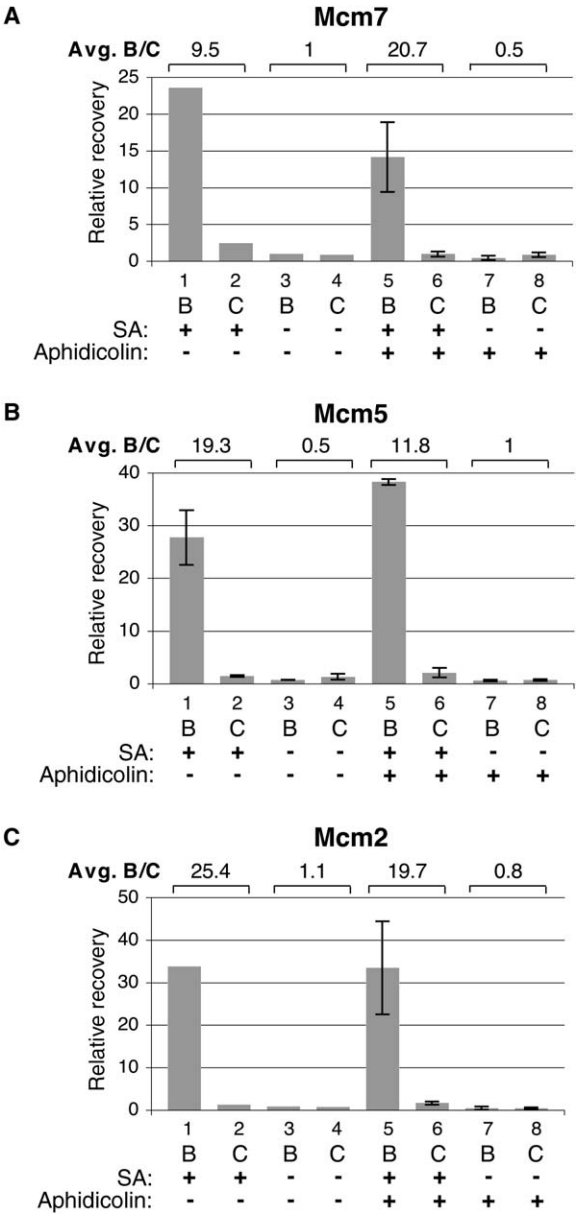


Figure 2. Mcm2, Mcm5, and Mcm7 Are Enriched at the Biotin-SA Locus in the Absence and Presence of Aphidicolin

ChIP was performed using Mcm7 (A), Mcm5 (B), or Mcm2 (C) antibodies. In each case, p2.6$^{Bio}$ (±SA) was incubated with HSS, and samples were processed for ChIP 10–15 min after NPE addition. NPE contained vehicle or aphidicolin, as indicated. For each condition, the relative recovery of *B*iotin and *C*ontrol loci in the immunoprecipitated material relative to the input material was determined by real-time PCR, and the average was graphed. In this and all other figures, error bars indicate minimum and maximum values. The average B/C ratio was calculated and indicated at the top of the graph.

Mcm5, and Mcm7 are part of a helicase complex that becomes uncoupled from the site of DNA synthesis when polymerases are inhibited by aphidicolin.

We next addressed whether aphidicolin causes MCM proteins to become uncoupled not only from the site of synthesis but also from DNA polymerases themselves. In the absence of aphidicolin, DNA pol α, DNA pol δ, and DNA pol ε were all enriched at the biotin-SA locus (Figures 3A–3C, columns 1 and 2). In contrast, in the presence of aphidicolin, DNA polymerases were not highly enriched at the biotin-SA locus (Figures 3A–3C, compare columns 5 and 6). The binding of DNA pol α was increased significantly by aphidicolin (Figure 3C, compare columns 1–4 with 5–8), consistent with the aphidicolin-induced hyperloading of DNA pol α previously reported in sperm chromatin spin-down experiments (e.g., Mimura et al. [2000]). In contrast, aphidicolin did not cause hyperloading of DNA pol ε or DNA pol δ (Figures 3A and 3B), consistent with sperm chromatin binding experiments (Mimura et al., 2000). However, these polymerases still bound the plasmid in the presence of aphidicolin, since the relative recovery of the B and C loci in the IP samples was on average 3.4-fold greater than in mock-IP controls (Table S1). Such a modest enrichment at the B and C loci (under conditions in which no pausing is expected) is typical of all the replication factors we examined (Table S1) and likely reflects the fact that only one or two copies of these proteins bind to each plasmid. The only exceptions are DNA pol α in the presence of aphidicolin (due to hyperloading), and MCM proteins at the early time points (because multiple MCM2-7 complexes are expected to load onto each template). We infer that, in the presence of aphidicolin, DNA polymerases ε and δ are bound at normal levels to the plasmid, but they are randomly distributed because they become arrested after loading, a process that is sequence independent. In summary, the data suggest that inhibition of DNA replication with aphidicolin causes physical uncoupling of the MCM2-7 complex from DNA polymerases and the site of DNA synthesis.

Several lines of genetic and biochemical evidence strongly suggest that Cdc45 is required for the activity of the replicative DNA helicase (see Introduction). To further test this idea, we examined Cdc45 using ChIP. Cdc45 was highly enriched at the biotin-SA locus in the absence of aphidicolin (Figure 4A, columns 1 and 2) as well as in its presence (Figure 4A, columns 5 and 6). Therefore, Cdc45 is not only associated with paused replisomes but also with the paused, uncoupled DNA helicase.

GINS is a four-subunit assembly consisting of Sld5, Psf1, Psf2, and Psf3 whose properties suggest it could be a helicase cofactor (see Introduction). Using ChIP, we found that Sld5 is enriched at the biotin-SA locus, in the absence of aphidicolin, indicating GINS is a component of the paused replisome (Figure 4B, columns 1 and 2). Strikingly, in the presence of aphidicolin, Sld5 was also highly enriched at the biotin locus, strongly suggesting that GINS is a component of the helicase complex (Figure 4B, columns 5 and 6).

Another potential candidate for a DNA helicase cofactor is Mcm10. However, we found that Mcm10 was enriched at the biotin locus in the absence of aphidicolin but not in its presence (Figure 4C, compare columns 1

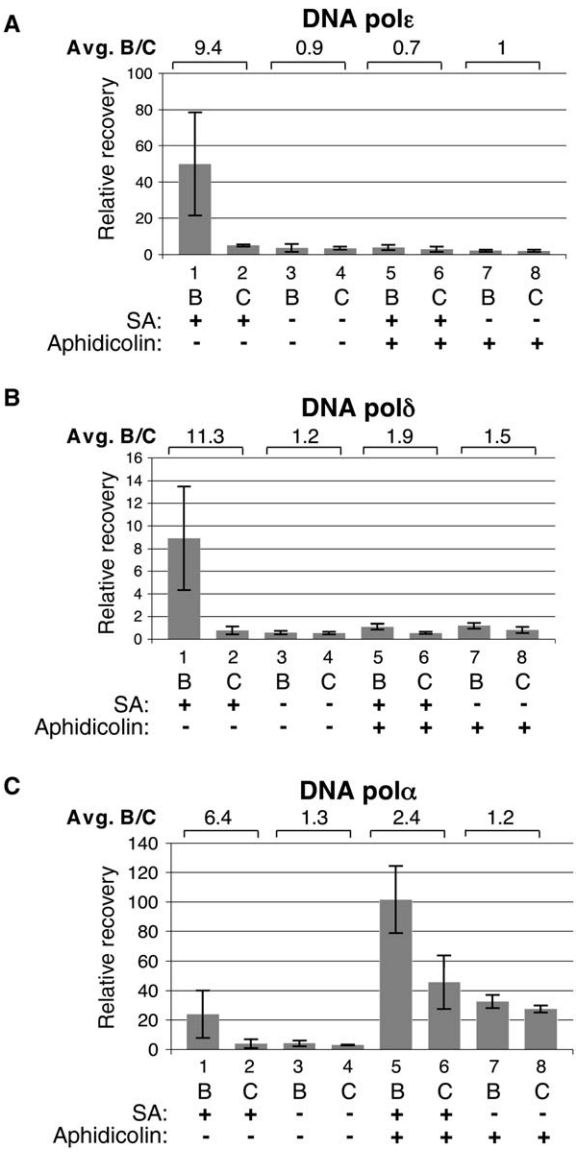


Figure 3. DNA Polymerases Are Enriched at the Biotin-SA Locus in the Absence of Aphidicolin but Not in Its Presence

ChIP was performed as in Figure 2A using antibodies against the 60 kDa subunit of DNA pol ε (A), the 66 kDa subunit of DNA pol δ (B), or the 70 kDa subunit of DNA pol α (C).

and 2 with 5 and 6), as seen also for DNA polymerases (Figure 3). Consistent with sperm chromatin-spin-down experiments (Wohlschlegel et al., 2002), Mcm10 still bound the plasmid in the presence of aphidicolin, since the Mcm10 IP recovered on average 3.2-fold more B and C DNA than a control IP (Table S1). Interestingly, unlike DNA pol α (Figure 3C), Mcm10 was not hyperloaded in the presence of aphidicolin (Figure 4C), suggesting that the additional DNA pol α loaded is not in a complex with Mcm10. We note that the average recovery of DNA using some antibodies (such as α-Mcm10) was lower than for others (such as α-Cdc45), but this is unlikely to cause a systematic error since there is no reason to expect that proteins crosslinked to the B locus should be more or less efficiently precipitated than proteins bound to the C locus. We conclude that Mcm10 is

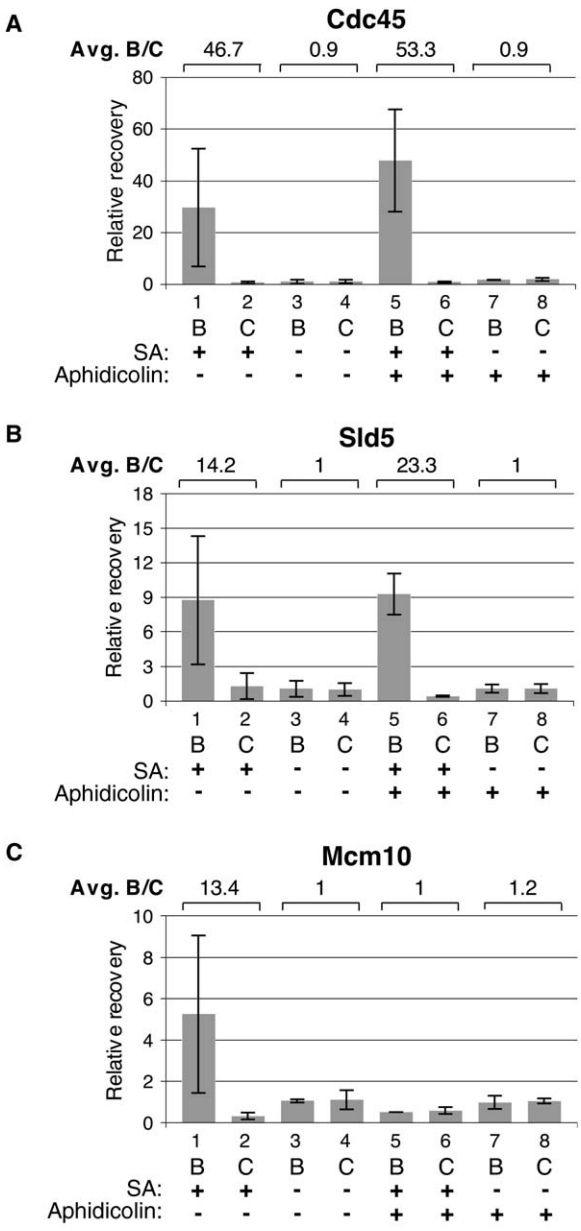


Figure 4. Cdc45 and GINS, but not MCM10, Are Enriched at the Biotin-SA Locus in the Presence of Aphidicolin

ChIP was performed as in Figure 2A using antibodies against Cdc45 (A), Sld5 (B), or Mcm10 (C).

a component of the intact replisome but not the uncoupled DNA helicase.

A potential concern in our ChIP experiments is that factors bind de novo to the replisome or helicase when these complexes undergo pausing. In the case of MCM2-7, we discount this possibility, since MCM2-7 loading in NPE is inhibited by geminin and the destruction of Cdt1 (Arias and Walter, 2005). In the case of Cdc45, Mcm10, and GINS, this possibility also appears remote because these proteins normally load onto chromatin in the absence of replicative stress, they persist on chromatin throughout S phase, and their binding is not affected positively or negatively by aphidicolin (Kubota et al., 2003; Mimura and Takisawa, 1998; Wohlschlegel et al., 2002). Nevertheless, we used caffeine to determine whether the enrichment of Cdc45 or Sld5 at the biotin-SA locus was dependent on the S phase checkpoint that is triggered by addition of aphidicolin, and we found that it was not (Figure S3).

In conclusion, we have developed a method to study replication elongation complexes in vertebrates. By inducing pausing of replication complexes at a defined site on a plasmid, ChIP can be used to interrogate their biochemical composition. MCM2-7, Cdc45, GINS, Mcm10, DNA pol $\alpha$, DNA pol $\delta$, and DNA pol $\varepsilon$ are found to localize to the vertebrate DNA replication fork. In the presence of aphidicolin, DNA polymerases and Mcm10 appear to remain bound to the chromatin but are not significantly enriched at the biotin locus, whereas MCM2-7, Cdc45, and Sld5 are highly enriched at the pause site, indicating that a helicase complex is physically uncoupled from the replisome. These data represent the first direct demonstration that MCM2-7, Cdc45, and GINS proteins are associated with the active helicase complex during DNA replication in higher eukaryotes. Consistent with our results, Cdc45, GINS, and MCM2-7 can be coprecipitated from chromatin in *Xenopus* egg extracts (Kubota et al., 2003), and a complex of these factors that exhibits helicase activity has been extensively purified from *Drosophila* eggs (S. Moyer and M. Botchan, personal communication). Our data argue that the MCM2-7 holocomplex participates in DNA unwinding, since members of all existing subcomplexes (Mcm4/6/7, Mcm3/5, and Mcm2) are present. Therefore, immunofluorescence data, which show a lack of colocalization of MCM subunits with sites of DNA replication, appear to be misleading. The requirement for Cdc45 and GINS during replication elongation (Kanemaki et al., 2003; Pacek and Walter, 2004; Tercero et al., 2000) suggests that their presence in the helicase complex reported here reflects essential auxiliary roles in DNA unwinding, although the mechanism remains to be elucidated. Our observations may explain why the MCM2-7 complex by itself exhibits no helicase activity in vitro and why the MCM4/6/7 complex unwinds DNA with low or moderate processivity. We propose to call the molecular machine that unwinds DNA at the replication fork the unwindosome, and we suspect it will contain factors other than Cdc45, GINS, and MCM2-7.

## Experimental Procedures

### Preparation of p2.6$^{Bio}$

A 2.6 kb PCR fragment was amplified using NcoI site-containing primers, one of which was also internally biotinylated using Bio-dT modification (Operon). The PCR product (~1 mg) was digested with NcoI, purified over a PD-10 column, and ligated in 7.5 mls. The closed circular form of the plasmid was isolated by electrophoresis on a 0.8% agarose gel containing 20 $\mu$M chloroquine. The yield of closed circular p2.6$^{Bio}$ was ~10 $\mu$g. The sequences of PCR primers and templates are available upon request.

### *Xenopus* Egg Extracts, DNA Replication, and 2D Gels

DNA replication and HSS and NPE preparation were as described (Walter et al., 1998). Briefly, plasmid DNA was incubated with HSS for 30 min (40 ng/$\mu$l final concentration) followed by addition of two volumes of NPE. For 2D gel analysis, NPE contained $^{32}$P [$\alpha$] dATP, and the reaction was stopped 10 min after NPE addition with 1% SDS and 1 mM EDTA, treated with RNase H and Proteinase K, followed by phenol/chloroform extraction. The DNA was digested with XbaI and MscI, separated using neutral-neutral 2D gel analysis

(Brewer and Fangman, 1987), and visualized using a phosphorimager.

### Chromatin Immunoprecipitation

$p2.6^{Bio}$ was preincubated with streptavidin or buffer for 1 hr at RT and subjected to DNA replication. ChIP was adopted from existing procedures (Danis et al., 2004; Harvey and Newport, 2003): after an incubation in NPE (10–15 min, depending on the efficiency of the extract), which allows ~70% of input DNA to be replicated (see Figure S1A), the reaction (30 μl) was diluted with 470 μl formaldehyde solution (1% formaldehyde in ELB salts, 2.5 mM $MgCl_2$, 10 mM HEPES [pH 7.7], 50 mM KCl) and incubated for 10 min at room temperature (RT). The crosslinked material was diluted to 2 mls with ELB (ELB salts + 250 mM sucrose) + 0.5% Triton X-100 and centrifuged immediately at 100,000 × g for 10 min in a Beckman TL-100 swinging bucket rotor. The pellet was washed with 2 ml of ELB + 0.5% Triton X-100. After recentrifugation as above, the pellet was resuspended in 650 μl sonication buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 2 mM PMSF, 1 mM EDTA, 0.5% NP-40, 5 μg/ml aprotein/leupeptin) and sheared to ~300 bp average size in a water-bath sonicator with ten 15" cycles of 35 W each. The sample was precleared for 1 hr with 30 μl of protein A Sepharose (PAS, Pharmacia). Subsequently, the sample was divided into four equal aliquots, which were left untreated (Input, Mock) or supplemented with one of two antibodies (IP samples). After overnight incubation at 4ºC, 10 μl PAS was added (Mock and IP samples) for 2 hr. Beads were washed three times with sonication buffer, three times with sonication buffer + 0.5 M NaCl, three times with wash buffer (10 mM Tris [pH 8.0], 0.5% Triton X-100, 1 mM EDTA), and once with TE. The beads, or the input sample, were diluted to 300 μl with elution buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 1%SDS) + RNase A for 30 min at 37ºC followed by 65ºC for 20 min. The supernatant was diluted 2-fold with TE and incubated with Pronase (1.5 μg/μl, Roche) for 6 hr at 42ºC followed by 9 hr at 72ºC. The samples were phenol/chloroform extracted and ethanol precipitated.

The DNA was diluted on average 400 times, and 5 μl was used in quantitative real-time PCR using the Applied Biosystems 7700 sequence detector based on SYBR Green fluorescence according to the manufacturer's protocol. PCR reactions using biotin or control locus primers and input, IP, or mock DNA as template were performed in triplicate on 96-well plates, and an average cycle threshold (CT) value was calculated for each reaction. To determine the amount of target DNA in the IP sample relative to the Input sample (a quantity we refer to as "X"), we used the equation $1.9^{[CT(IP) - CT(Input)]}$, where CT(IP) and CT(Input) are the CT values for a given locus in the IP and Input materials, respectively. The amount of target DNA in the input was arbitrarily assigned a value of 100, and the "relative recovery" of DNA in the IP sample relative to the input was 100/X. The enrichment of B over C was calculated by dividing the relative recovery of B by the relative recovery of C in each experiment.

To determine the background ("Avg. Mock" in Supplemental Data tables), we calculated the amount of target DNA in the mock-IP sample relative to the Input sample using the equation $1.9^{[CT(mock) - CT(Input)]}$, and we called this value "Y." The recovery of DNA in the mock-IP relative to input was 100/Y.

ChIPs were performed using 1 μl of rabbit serum raised against Mcm7 (Walter and Newport, 2000), Cdc45 (Walter and Newport, 2000), the 66 kDa subunit of DNA pol δ (Fukui et al., 2004), the 70 kDa subunit of DNA pol α (Arias and Walter, 2005), or the 60 kDa subunit of DNA pol ε (Waga et al., 2001); 0.5 μg of affinity-purified antibody against Sld5; or 2 μg of purified peptide antibody against Mcm2 (Bethyl laboratories), Mcm5 (Bethyl laboratories), or Mcm10 (A.V.T. and J.C.W., unpublished data).

### Supplemental Data

Supplemental Data include three figures and three tables and can be found with this article online at http://www.molecule.org/cgi/content/full/21/4/581/DC1/.

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