

A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication

Marcin Pacek and Johannes C Walter*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

In vertebrates, MCM2–7 and Cdc45 are required for DNA replication initiation, but it is unknown whether they are also required for elongation, as in yeast. Moreover, although MCM2–7 is a prime candidate for the eukaryotic replicative DNA helicase, a demonstration that MCM2–7 unwinds DNA during replication is lacking. Here, we use *Xenopus* egg extracts to investigate the roles of MCM7 and Cdc45 in DNA replication. A fragment of the retinoblastoma protein, Rb^{1–400}, was used to neutralize MCM7, and antibodies were used to neutralize Cdc45. When added immediately after origin unwinding, or after significant DNA synthesis, both inhibitors blocked further DNA replication, indicating that MCM7 and Cdc45 are required throughout replication elongation in vertebrates. We next exploited the fact that inhibition of DNA polymerase by aphidicolin causes extensive chromosome unwinding, likely due to uncoupling of the replicative DNA helicase. Strikingly, Rb^{1–400} and Cdc45 antibodies both abolished unwinding by the uncoupled helicase. These results provide new support for the model that MCM2–7 is the replicative DNA helicase, and they indicate that Cdc45 functions as a helicase co-factor.

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Introduction

Vertebrate cells contain a vast amount of DNA that is faithfully replicated during every mitotic cell division (for reviews, see Waga and Stillman, 1998; Bell and Dutta, 2002; Mendez and Stillman, 2003). The first step in DNA replication, pre-RC assembly, occurs at thousands of origins during the G1 phase of the cell cycle when a six-subunit origin recognition complex (ORC) bound to origins recruits the initiation factors Cdc6, Cdt1, and MCM2–7. Once MCM2–7 is loaded, ORC becomes dispensable, and the MCM2–7 complex serves as

the platform on which further initiation events take place (Hua and Newport, 1998; Rowles *et al.*, 1999; Shimada *et al.*, 2002).

In S phase, pre-RCs are acted upon by two protein kinases and a multitude of replication initiation factors, which together are required for origin unwinding. In metazoans, MCM10 and the protein kinase Cdc7/Dbf4 are the first factors to load onto pre-RCs, and their loading is MCM2–7-dependent (Jares and Blow, 2000; Walter, 2000; Wohlschlegel *et al.*, 2002). MCM10 and Cdc7 enable the loading of several additional factors such as GINS and Cdc45, whose binding is also dependent on Cdk2/Cyclin E. The binding of Cdc45 and GINS to pre-RCs is interdependent and converts this structure into a pre-Initiation Complex (or pre-IC) (Mimura and Takisawa, 1998; Zou and Stillman, 1998, 2000; Jares and Blow, 2000; Walter, 2000; Kubota *et al.*, 2003; Takayama *et al.*, 2003). Formation of the pre-IC is the last known event that occurs before origin unwinding, which is accompanied by chromatin loading of the single-stranded DNA-binding protein RPA (Tanaka and Nasmyth, 1998; Mimura *et al.*, 2000; Walter and Newport, 2000). Once the origin has been sufficiently unwound, DNA polymerase α loads and synthesizes an RNA primer, which it then extends to form a short DNA primer. The presence of a DNA primer allows loading of the processivity factor PCNA by the RFC complex, followed by pol δ .

The final stage of DNA replication, elongation, involves the coordinated synthesis of nascent strands. Studies in yeast clearly show that, in addition to DNA polymerases, elongation also requires MCM2–7, Cdc45, and GINS (Labib *et al.*, 2000; Tercero *et al.*, 2000; Kanemaki *et al.*, 2003), all of which localize to replication forks (Aparicio *et al.*, 1997; Kanemaki *et al.*, 2003; Takayama *et al.*, 2003). It is unknown whether MCM2–7, Cdc45, or GINS are also required for elongation in metazoans. Given the results in yeast, it is surprising that immunofluorescence studies in vertebrate cells failed to detect colocalization of MCM2–7 with sites of ongoing DNA replication (Todorov *et al.*, 1994; Krude *et al.*, 1996; Romanowski *et al.*, 1996).

The requirement for MCM2–7 in elongation in yeast is consistent with it being the eukaryotic replicative DNA helicase (Labib and Diffley, 2001). Like the replicative DNA helicases DnaB and Large T antigen, all six MCM subunits are members of the AAA+ family of ATPases, and the MCM2–7 complex adopts a ring-like structure (Chong *et al.*, 2000; Fletcher *et al.*, 2003). In yeast and mammals, a purified MCM4/6/7 subcomplex exhibits helicase activity in oligonucleotide displacement assays (You *et al.*, 1999; Lee and Hurwitz, 2000; Kaplan *et al.*, 2003). However, MCM2–7 is inactive as a helicase, and the purified MCM4/6/7 complex is inhibited by MCM2 and MCM3/5 (Ishimi *et al.*, 1998; Lee and Hurwitz, 2000), which is surprising given the requirement for all six MCM subunits in fork progression *in vivo* (Labib *et al.*, 2000). These observations have led to models in which MCM4/6/7 is the motor that unwinds DNA, whereas the

*Corresponding author. Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, BCMP, C2-Room 322, 240 Longwood Avenue, Boston, MA 02115, USA. Tel.: +1 617 432 4799; Fax: +1 617 738 0516; E-mail: johannes_walter@hms.harvard.edu

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other subunits serve regulatory functions. A definitive demonstration that MCM2-7 is the eukaryotic replicative DNA helicase will require biochemical reconstitution of DNA replication or a demonstration that MCM2-7 performs helicase activity at the replication fork (Labib and Diffley, 2001). Unlike the MCM2-7 complex, Cdc45 contains no known sequence motifs, and its molecular mechanism is unclear. Interestingly, Cdc45 is found in a complex with MCM2-7 on chromatin in yeast and in *Xenopus* egg extracts (Zou and Stillman, 1998; Mimura *et al.*, 2000), suggesting that it might regulate the activity of MCM2-7.

To study chromosomal DNA replication, we use a soluble cell-free system derived from *Xenopus* eggs. Sperm chromatin or plasmid DNA is first incubated in a high speed supernatant of egg cytoplasm (HSS), leading to pre-RC assembly. Subsequently, a highly concentrated nucleoplasmic extract (NPE) prepared from synthetic nuclei is added, which supplies Cdk2/Cyclin E and Cdc7/Dbf4, as well as other activities (Walter, 2000; Wohlschlegel *et al.*, 2002; Prokhorova *et al.*, 2003) and a complete round of DNA replication ensues. Using this system, origin unwinding can be detected on plasmids via negative supercoiling, or on sperm via RPA loading (Walter and Newport, 2000). Interestingly, in the presence of aphidicolin, which inhibits replicative DNA polymerases, the degree of negative supercoiling and RPA loading are dramatically enhanced, indicating a high degree of DNA unwinding, or 'hyperunwinding' (Walter, 2000; Walter and Newport, 2000). Since hyperunwinding depends on prior initiation of DNA replication, and is rapid and extensive, it likely reflects the action of the replicative DNA helicase after it has become uncoupled from the stalled replication complex. Thus, aphidicolin-induced helicase uncoupling represents a potentially powerful assay to study the eukaryotic replicative DNA helicase in the context of replication-competent chromatin.

We wanted to determine whether Cdc45 and the MCM complex are required after pre-IC formation in *Xenopus* egg extracts. To inactivate the chromatin-bound MCM complex after pre-ICs had formed, we used the N-terminal 400 amino acids of Rb (Rb¹⁻⁴⁰⁰), a domain that was previously shown to interact with MCM7 and thereby inhibit DNA replication in *Xenopus* egg extracts at an unknown step (Sterner *et al.*, 1998). When we added Rb¹⁻⁴⁰⁰ to DNA replication complexes synchronized immediately after origin unwinding or during elongation, it completely inhibited further DNA replication, indicating a role for MCM7 in elongation. Moreover, at both of these stages, Rb¹⁻⁴⁰⁰ inhibited the activity of the uncoupled DNA helicase in the presence of aphidicolin. These experiments show a direct role for MCM7 in DNA unwinding in the context of replication-competent chromatin, and thus provide new support for the idea that the MCM complex is the replicative DNA helicase. To determine whether Cdc45 is required for elongation, we inactivated chromatin-bound Cdc45 using antibodies. Cdc45 antibodies inhibited DNA replication and DNA unwinding by the uncoupled DNA helicase when added to replication complexes synchronized immediately after origin unwinding, or during elongation. The data show that Cdc45 is required for chromosome unwinding during elongation, and they are consistent with a model in which Cdc45 stimulates the helicase activity of the MCM2-7 complex.

Results

Rb¹⁻⁴⁰⁰ inhibits origin unwinding when added after pre-RC formation

To examine what steps of DNA replication after pre-IC formation are dependent on the MCM complex, we sought to inactivate the chromatin-bound complex at progressively later stages of DNA replication. To this end, we took advantage of the previous observation that the retinoblastoma gene product, Rb, binds to MCM7 (Sterner *et al.*, 1998). Using two-hybrid assays and co-immunoprecipitation of mammalian cell extracts, Rb¹⁻⁴⁰⁰ was found to interact with the C-terminal region of MCM7. Moreover, when Rb¹⁻⁴⁰⁰ was added to nuclear-assembly egg extracts before sperm chromatin, DNA replication was blocked. The inhibition was reversed when Rb¹⁻⁴⁰⁰ was pre-incubated with an MCM7 peptide, arguing that inhibition was the result of Rb¹⁻⁴⁰⁰ binding to the endogenous MCM7 protein (Sterner *et al.*, 1998). It was not determined which step in DNA replication is blocked by Rb¹⁻⁴⁰⁰.

We examined the effects of Rb¹⁻⁴⁰⁰ on DNA replication in the nucleus-free system (see Introduction). Initially, Rb¹⁻⁴⁰⁰ was added to HSS before sperm chromatin. Upon addition of NPE, DNA replication was blocked, and the inhibition was relieved by the MCM7 peptide (data not shown). Further analysis showed that, in the presence of Rb¹⁻⁴⁰⁰, the MCM complex and Cdc45 failed to load onto chromatin (data not shown). These results show that, when added before sperm chromatin, Rb¹⁻⁴⁰⁰ can block pre-RC assembly, and they confirm the previous observation that Rb¹⁻⁴⁰⁰ inhibits DNA replication in *Xenopus* egg extracts (Sterner *et al.*, 1998).

We next sought to determine what happens when Rb¹⁻⁴⁰⁰ is added after MCM2-7 complexes have loaded onto chromatin. Sperm chromatin was incubated in HSS to assemble pre-RCs. Subsequently, Rb¹⁻⁴⁰⁰ was added, followed by NPE. Under these conditions, Rb¹⁻⁴⁰⁰ inhibited DNA replication five-fold, but when Rb¹⁻⁴⁰⁰ was pre-incubated with MCM7 peptide, inhibition was relieved (Figure 1A, bar graph). To determine at what stage DNA replication was blocked, chromatin was isolated from NPE containing aphidicolin (NPE^{aph}) to examine which factors loaded in the presence of Rb¹⁻⁴⁰⁰. As discussed in the Introduction, the hyperloading of RPA in the presence of aphidicolin likely reflects the action of the replicative DNA helicase after it has become uncoupled from the stalled replication complex. In the presence of Rb¹⁻⁴⁰⁰, MCM7, ORC2, and Cdc45 binding was unaffected, but RPA hyperloading was reduced (Figure 1A, upper panel, compare lanes 1 and 2), and the effect was reversed when Rb¹⁻⁴⁰⁰ was preincubated with MCM7 peptide (Figure 1A, lane 3). Importantly, Rb¹⁻⁴⁰⁰ did not affect the binding of RPA to an immobilized single-stranded DNA oligonucleotide in extracts (data not shown), indicating that the inhibition of RPA loading by Rb¹⁻⁴⁰⁰ was due to an effect on chromosome unwinding.

To confirm by another method that Rb¹⁻⁴⁰⁰ inhibits origin unwinding when added to assembled pre-RCs, we used a DNA topology assay (Walter and Newport, 2000). DNA replication is carried out using a circular plasmid, such as pBluescript (pBS), as the DNA template. Upon initiation of DNA replication, the plasmid becomes transiently underwound, generating a negatively supercoiled species that is readily detected by its rapid mobility during electrophoresis

(U-form DNA). Addition of aphidicolin to the NPE traps all the DNA in the U form, and each plasmid undergoes much more extensive unwinding ('hyperunwinding') than in the absence of aphidicolin. Hyperunwinding in the presence of

aphidicolin is sensitive to the Cdk2 inhibitor p27^{Kip} (Walter and Newport, 2000; Figure 1B, upper panel, compare lanes 1 and 2), indicating that it reflects uncoupling of the replicative DNA helicase after initiation. To determine whether Rb¹⁻⁴⁰⁰ affected origin unwinding when added after pre-RC formation, pBS was incubated in HSS to form pre-RCs. Subsequently, Rb¹⁻⁴⁰⁰ was added, followed by NPE containing aphidicolin. Rb¹⁻⁴⁰⁰ significantly reduced the generation of U-form DNA (Figure 1B, lanes 1 and 3), and inhibition was largely alleviated when Rb¹⁻⁴⁰⁰ was pre-incubated with MCM7 peptide (Figure 1B, lane 4). Consistent with the inhibition of origin unwinding, Rb¹⁻⁴⁰⁰ also blocked DNA replication of pBS when it was added after pre-RC formation (Figure 1B, bar graph).

We wanted to verify that Rb¹⁻⁴⁰⁰ targeted MCM7 on chromatin. Sperm chromatin was incubated with HSS to load the MCM complex, followed by addition of Rb¹⁻⁴⁰⁰. Subsequently, the chromatin was isolated and blotted for Rb. Figure 1C (lane 1) shows that Rb¹⁻⁴⁰⁰ cosedimented with the chromatin. Importantly, in the presence of geminin, which blocks MCM2-7 chromatin loading by targeting Cdt1 (Wohlschlegel *et al*, 2000; Tada *et al*, 2001), Rb¹⁻⁴⁰⁰ loading was reduced (Figure 1C, lane 2). When Rb¹⁻⁴⁰⁰ was pre-incubated with MCM7 peptide, Rb¹⁻⁴⁰⁰ binding was also severely reduced (Figure 1C, lane 3). These results argue that Rb¹⁻⁴⁰⁰ exerts its inhibitory effects in DNA replication and origin unwinding by binding to MCM7 on chromatin. Together, the data in this section indicate that MCM7 is required for origin unwinding independently of its role in pre-IC formation.

Rb¹⁻⁴⁰⁰ inhibits chromosome unwinding by a previously activated helicase

The origin-unwinding defect seen in Figure 1 could be explained in two ways. First, Rb¹⁻⁴⁰⁰ might block an unknown step after pre-IC formation that is required to activate the replicative DNA helicase, or Rb¹⁻⁴⁰⁰ might block the fully activated helicase itself. To distinguish between these possibilities, we added Rb¹⁻⁴⁰⁰ to chromatin containing a

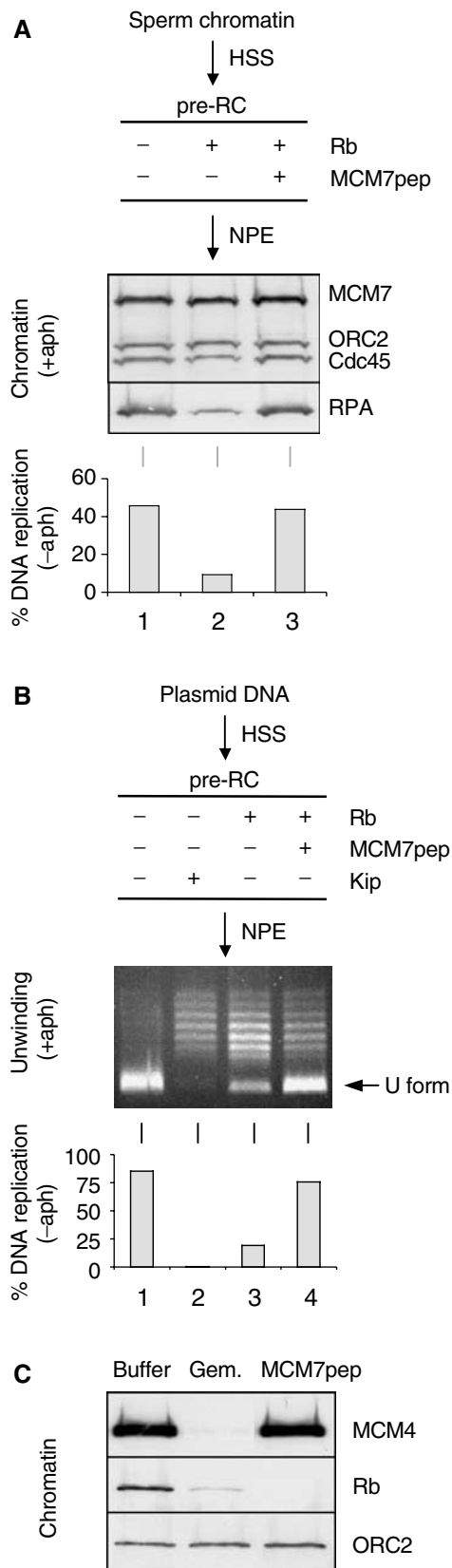


Figure 1 Rb¹⁻⁴⁰⁰ inhibits origin unwinding after pre-RC formation. (A) Chromatin-loading assay. Sperm chromatin (10 000/μl) was incubated with 2 μl of HSS for 30 min and then supplemented with buffer (lane 1), 800 ng Rb¹⁻⁴⁰⁰ (lane 2), or 800 ng Rb¹⁻⁴⁰⁰ preincubated with 1.6 μg MBP-MCM7 peptide fusion (lane 3). After 30 min, 4 μl NPE was added, which contained aphidicolin (50 μg/ml) and buffer (lane 1), 400 ng Rb¹⁻⁴⁰⁰ (lane 2), or 400 ng Rb¹⁻⁴⁰⁰ and 800 ng MCM7 peptide (lane 3). After 45 min, the chromatin was purified, and bound proteins were analyzed by Western blot analysis using antibodies against RPA (lower panel), and a mixture of antibodies against MCM7, ORC2, and Cdc45 (upper panel). To measure DNA replication, the same reaction was carried out using NPE lacking aphidicolin but containing [α -³²P]dATP (lower panel). (B) DNA topology assay. Same as panel A, except that pBS (40 ng/μl) was used as the DNA template. Lane 2 shows the effect of p27^{Kip} addition. After 30 min incubation with NPE, the DNA was extracted, separated on a chloroquine agarose gel, and stained (top panel). To measure DNA replication, the same reaction was carried out using NPE lacking aphidicolin but containing [α -³²P]dATP (lower panel). (C) Sperm chromatin was incubated with HSS supplemented with control buffer (lanes 1 and 3), or 500 nM geminin (lane 2) for 30 min. Subsequently, buffer (lane 1), 800 ng Rb¹⁻⁴⁰⁰ (lane 2), or 800 ng Rb¹⁻⁴⁰⁰ preincubated with 1.6 μg MCM7 peptide (lane 3) was added. After further 30 min, chromatin-bound proteins were analyzed with antibodies against MCM4, Rb, or ORC2.

previously activated helicase. To this end, sperm chromatin was incubated with HSS, followed by NPE containing actinomycin D (NPE^{actD}). ActD abolishes DNA replication in *Xenopus* egg extracts (Michael *et al.*, 2000; our data not shown), likely due to its inhibition of the RNA priming activity of DNA pol α (Grosse and Krauss, 1985). Consistent with such a mechanism, actD does not affect chromatin loading of Cdc45 (Edwards *et al.*, 2002) or DNA polymerase α (data not shown). Importantly, in the presence of actD, RPA was also loaded onto chromatin, and the loading was Cdk2-dependent, demonstrating that it required initiation (Figure 2A, compare lanes 5 and 6). The amount of RPA loaded in the presence of actD was similar to the peak level seen on chromatin during an *unperturbed* S phase (Figure 2A, compare lanes 2 and 5), indicating that unwinding in NPE^{actD} was significant, but it was far less than what is observed during hyperunwinding in aphidicolin extract (Figure 2A, compare lanes 5 and 7). The reason why actD does not support hyperunwinding is unclear. One possibility is that its intercalating activity (Kamitori and Takusagawa, 1992) prevents the ability of the helicase to travel more than a short distance along DNA. Whatever the precise mechanism of actD, the data show that it arrests DNA replication after a Cdk2-dependent helicase has been allowed to unwind a limited amount of DNA (Figure 2B, top).

When chromatin containing the activated helicase was transferred from NPE^{actD} to fresh NPE, DNA replication was efficient, indicating that a replication complex stalled in actD can resume DNA synthesis (Figure 2C, bar graph, column 2). When the chromatin incubated in NPE^{actD} was first exposed to Rb¹⁻⁴⁰⁰ before transfer to fresh NPE, DNA replication was severely inhibited, but this inhibition was not observed when Rb¹⁻⁴⁰⁰ was first pre-incubated with MCM7 peptide (Figure 2C, bar graph, compare columns 3 and 4). Therefore, MCM7 is still required for DNA replication after helicase activation. The experiment was repeated, but chromatin was transferred from NPE^{actD} into NPE^{aph}, and chromatin association of RPA was measured. There was a large increase in RPA binding when chromatin was transferred from NPE^{actD} into NPE^{aph} (Figure 2C, upper panel, compare lanes 1 and 2). This increase was dependent on the presence of aphidicolin because it did not occur when chromatin was transferred into fresh NPE^{actD} (Supplementary Figure S1). Moreover, it did not involve new initiation events because

it still occurred when chromatin was transferred into NPE^{aph} that also contained the Cdk2 inhibitor, p27^{Kip} (Supplementary Figure S1). Therefore, a helicase arrested in the presence of actD can subsequently become uncoupled from the replication fork and carry out hyperunwinding in the presence of aphidicolin (illustrated in Figure 2B). When chromatin incubated in NPE^{actD} was exposed to Rb¹⁻⁴⁰⁰ before transfer into NPE^{aph}, RPA hyperloading was strongly reduced, and the effect was reversed by MCM7 peptide (Figure 2C, upper

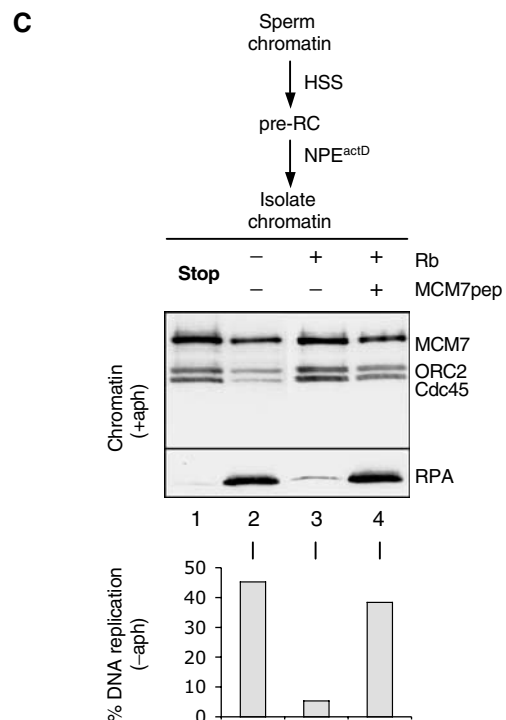
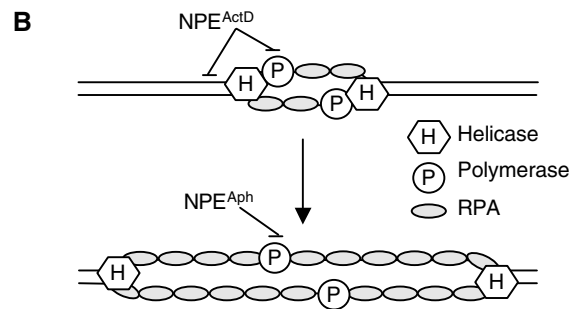
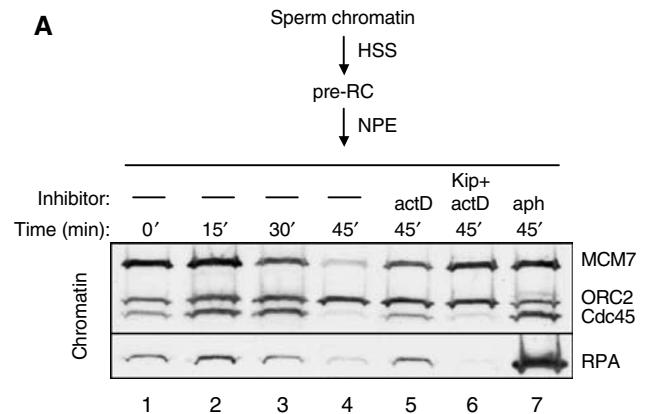


Figure 2 Rb¹⁻⁴⁰⁰ protein inhibits DNA replication and chromosome unwinding after initiation. (A) RPA loading in the presence of actinomycin D is Cdk2-dependent. Sperm chromatin was incubated with HSS, followed by unsupplemented NPE (lanes 1–4), or NPE containing 10 μ M actinomycin D (lanes 5), actinomycin D and p27^{Kip} (lane 6), or aphidicolin (lane 7). At the indicated times, chromatin was isolated and blotted for MCM7, ORC2, Cdc45, and RPA. (B) Model for the actinomycin D and aphidicolin arrest points. (C) Sperm chromatin was incubated with HSS, followed by NPE^{actD}, and isolated (lane 1), or isolated and then incubated with buffer (lane 2), 800 ng Rb¹⁻⁴⁰⁰ (lane 3), or 800 ng Rb¹⁻⁴⁰⁰ preincubated with 1.6 μ g MCM7 peptide. Isolation leads to permanent immobilization of the sperm on the tube. After 30 min, the supernatant was replaced with 5 μ l fresh NPE^{aph} containing buffer (lane 2), 400 ng Rb¹⁻⁴⁰⁰ (lane 3), or 400 ng Rb¹⁻⁴⁰⁰ preincubated with 800 ng MCM7 peptide. After 45 min, chromatin was washed and blotted for MCM7, ORC2, Cdc45, and RPA34 (upper panel). Identical reactions were carried out in which the second incubation with NPE lacked aphidicolin but contained [α -³²P]dATP to measure DNA replication (bar graph).

panel, lanes 3 and 4). The data indicate that, even after the replicative DNA helicase has been activated, DNA replication still requires MCM7 due to a direct involvement of this protein in chromosome unwinding.

Rb¹⁻⁴⁰⁰ inhibits DNA replication and chromosome unwinding during replication elongation

Although there is origin unwinding of DNA templates incubated in NPE^{actD} (Figure 2A), there is no DNA synthesis (Michael *et al*, 2000; our data not shown). Therefore, replication complexes assembled in these extracts have not entered the elongation phase of DNA replication. To address whether Rb¹⁻⁴⁰⁰ inhibits DNA replication of chromatin engaged in elongation, we synchronized replication complexes in elongation mode by lowering the reaction temperature from 22 to 19°C, which caused S phase to be extended by at least 30 min (Figure 3A). To determine the synchrony of origin firing at 19°C, we added p27^{Kip} 20 min after the addition of NPE and found that it had no effect on the kinetics or efficiency of DNA replication (Figure 3B, compare circles and triangles), whereas when p27^{Kip} was added at the same time as NPE DNA replication was completely blocked (Figure 3B, squares). We know that Cdk2 remained inactive throughout the experiment because a fresh, licensed template added after p27^{Kip} did not undergo DNA replication (data not shown). The data indicate that, at 19°C, all origins fire within 20 min of NPE addition, and the time following Cdk2 addition comprises the elongation phase of DNA replication.

We used the low-temperature synchronization to address whether Rb¹⁻⁴⁰⁰ inhibits DNA replication of elongating complexes. Pre-RCs were assembled in HSS and allowed to initiate DNA replication in NPE at 19°C. After 25 min, p27^{Kip} was added to inhibit additional initiation events, and after a further 15 min, the chromatin was isolated. At this stage, 30% of the input DNA had replicated (Figure 3C, bar graph, column 1). Individual aliquots of chromatin were then mixed with buffer, Rb¹⁻⁴⁰⁰, or Rb¹⁻⁴⁰⁰ and MCM7 peptide, followed by fresh NPE, which was supplemented with p27^{Kip} to prevent new initiation events. In the buffer control, addition of NPE^{Kip} allowed a further 20% DNA replication (Figure 3C, column 2). It should be noted that the relatively low efficiency of DNA replication observed after chromatin isolation is due to nonspecific inactivation of chromatin (Walter *et al*, 1998), but it does not affect our conclusions. In contrast, only ~1% further replication was observed in

the presence of Rb¹⁻⁴⁰⁰, and 16% additional DNA replication was seen in the presence of Rb¹⁻⁴⁰⁰ and MCM7 peptide (Figure 3C, columns 3 and 4). These results indicate that MCM7 is required for replication elongation.

To determine the effects of Rb¹⁻⁴⁰⁰ on chromosome unwinding by elongating complexes, chromatin was treated as described above, but it was incubated with NPE^{Kip+aph} in the final step to measure RPA hyperloading. As seen in Figure 3C (lower panel), transfer of elongating complexes into NPE^{Kip+aph} caused hyperloading of RPA, indicating that the

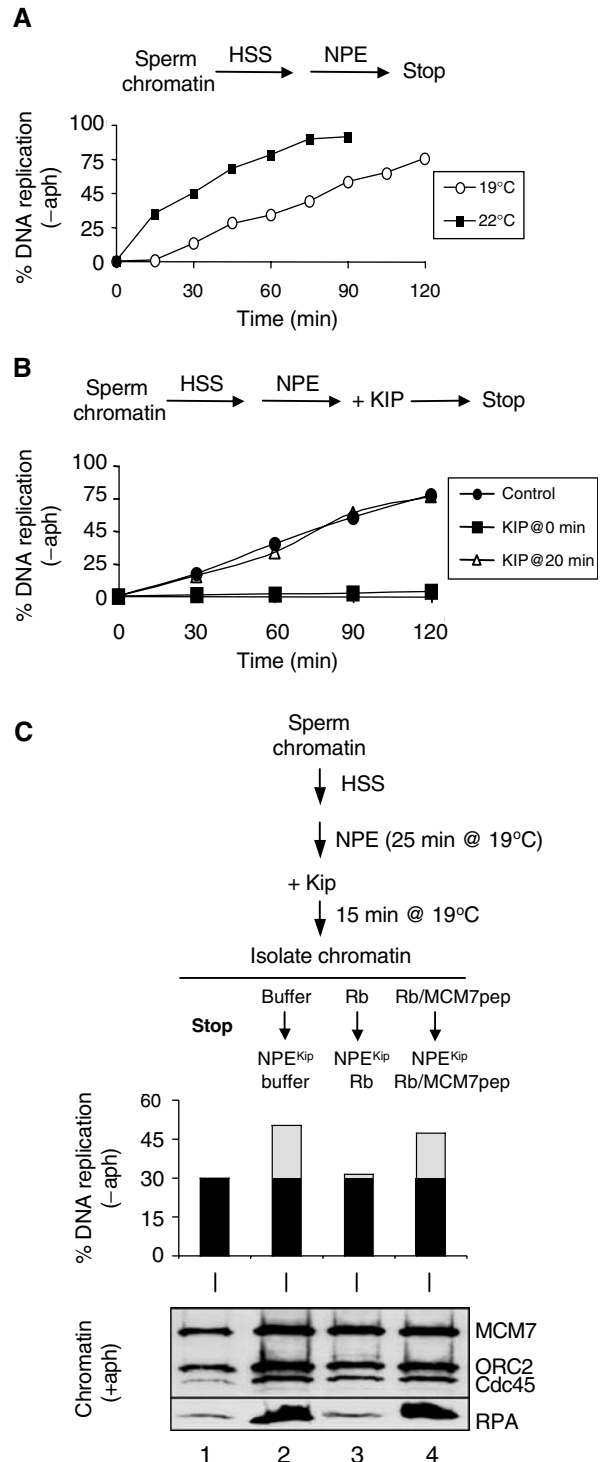


Figure 3 Rb¹⁻⁴⁰⁰ inhibits DNA replication and chromosome unwinding during elongation. (A) DNA replication kinetics at 22 and 19°C. Sperm chromatin was pre-incubated with HSS, NPE was added, reactions were transferred to 22°C (squares) or 19°C (circles), and DNA replication was measured. (B) All origins fire within 20 min of NPE addition at 19°C. Sperm chromatin was incubated with HSS, followed by NPE addition and transfer to 19°C. Buffer (circles) or p27^{Kip} was added 0 min (squares) and 20 min (triangles) after NPE. (C) Rb¹⁻⁴⁰⁰ inhibits the elongation complex. Sperm chromatin was incubated with HSS, followed by addition of NPE and transfer to 19°C. After 25 min, p27^{Kip} was added, and after a further 15 min chromatin was isolated (lane 1). Chromatin was exposed to buffer (lane 2), Rb¹⁻⁴⁰⁰ (lane 3), or Rb¹⁻⁴⁰⁰/MCM7 (lane 4). Finally, fresh NPE containing aphidicolin, p27^{Kip}, and buffer (lane 2), Rb¹⁻⁴⁰⁰ (lane 3), or Rb¹⁻⁴⁰⁰/MCM7 peptide (lane 4) was added to measure chromatin binding (lower panel). To measure DNA replication (bar graph), aphidicolin was omitted and both NPEs contained [α -³²P]dATP.

helicase can become uncoupled from elongating complexes (compare lanes 1 and 2). As with DNA replication, Rb¹⁻⁴⁰⁰ blocked RPA hyperloading, and the effect was reversed by MCM7 peptide (Figure 3C, lanes 3 and 4). The data show that Rb¹⁻⁴⁰⁰ inhibits DNA replication and chromosome unwinding of elongating replication complexes, consistent with the idea that MCM7 is directly required for chromosome unwinding in these complexes.

In Figure 3C, it was conceivable that Rb inhibited DNA replication of the isolated chromatin due to a requirement for MCM7 in restarting the replication fork upon transfer to fresh NPE. However, this scenario is unlikely, since Rb (but not Rb pre-incubated with MCM7 peptide) inhibited DNA replication when added to elongating complexes that had not been isolated from the extract (Supplementary Figure S2).

Previously, it was shown that multiple MCM2-7 complexes bind to chromatin in a distributed pattern in *Xenopus* egg extracts (Edwards *et al*, 2002; Harvey and Newport, 2003). Therefore, it was conceivable that Rb¹⁻⁴⁰⁰ might block DNA replication and chromosome unwinding by associating with multiple MCM2-7 complexes, thereby creating a chromatin structure that cannot be traversed by the replication complex. If this model were correct, chromatin with a lower density of MCM2-7 complexes should be impervious to inhibition by Rb¹⁻⁴⁰⁰. To generate such chromatin, we incubated sperm in HSS that was diluted 10-fold with buffer. Under these conditions, the amount of chromatin-bound MCM2-7 complexes was severely reduced compared to undiluted HSS (Figure 4A, compare lanes 1 and 3), but the residual MCM2-7 loading was still geminin-sensitive (Figure 4A, lane 4). To test whether Rb¹⁻⁴⁰⁰ still inhibits DNA replication of chromatin containing low levels of MCM2-7, we performed a low-temperature synchronization in which we used either undiluted or 10-fold diluted HSS. Figure 4B shows that DNA replication of chromatin assembled in dilute HSS was ~50% of control (compare columns 1 and 2), consistent with previous findings that MCM complexes are present on chromatin in a large functional excess (Mahbubani *et al*, 1997; Edwards *et al*, 2002), and replication was still completely geminin-sensitive (compare columns 2 and 4). Importantly, despite the low level of MCM2-7 bound, Rb¹⁻⁴⁰⁰ inhibited DNA replication of chromatin assembled in dilute HSS to the same extent as control chromatin (compare columns 1 and 5 with 2 and 6), and in each case MCM7 peptide rescued DNA replication (columns 7 and 8). This result argues against the idea that inhibition by Rb¹⁻⁴⁰⁰ results from its association with multiple MCM complexes to generate a repressive chromatin structure.

Cdc45 is required for DNA replication and chromosome unwinding after initiation

In Cdc45-depleted egg extracts, pre-IC formation is defective, and no origin unwinding is detected (Mimura and Takisawa, 1998; Mimura *et al*, 2000; Walter and Newport, 2000). To determine whether Cdc45 is required after pre-IC formation, we used a high-titer polyclonal antibody raised against the Cdc45 protein (Walter and Newport, 2000). When added to egg extracts before sperm chromatin, this antibody blocked Cdc45 loading and inhibited DNA replication (data not shown). To determine whether Cdc45 is still required after helicase activation, we incubated sperm chromatin in NPE^{actD}, which allows Cdc45 loading and limited chromo-

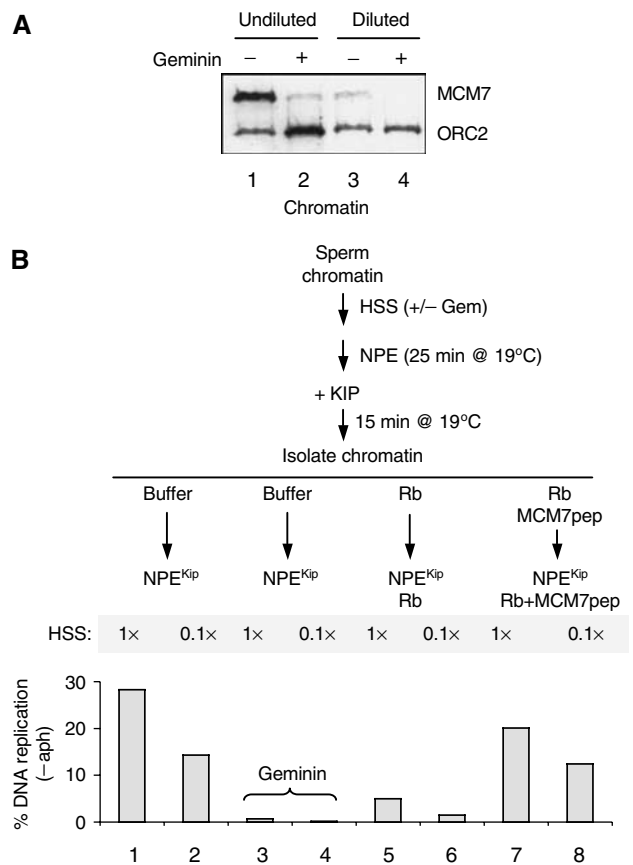


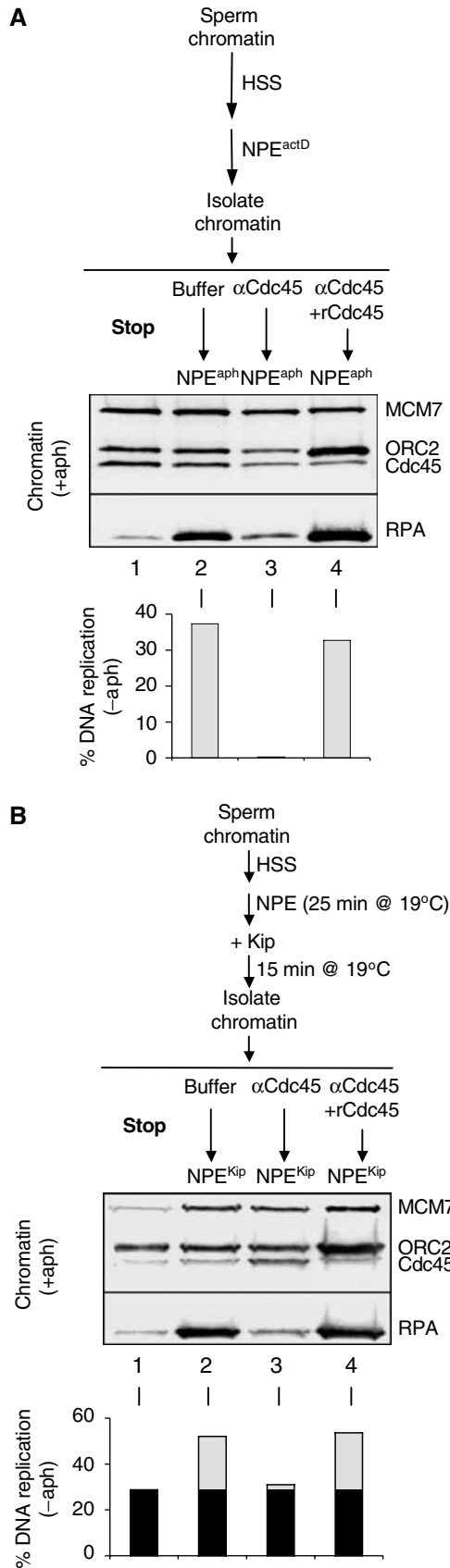
Figure 4 Rb¹⁻⁴⁰⁰ inhibits DNA replication of chromatin containing low levels of MCM2-7. **(A)** Effect of HSS dilution on MCM2-7 loading. Sperm chromatin was incubated with HSS (lanes 1 and 2), or HSS that was diluted 10-fold with ELB (lanes 3 and 4), in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of geminin. After 30 min, the chromatin was isolated and blotted for MCM7 and ORC2. **(B)** Effect of Rb¹⁻⁴⁰⁰ on chromatin containing low levels of MCM2-7. Sperm chromatin was incubated with HSS (1x) or 10 times diluted HSS (0.1x), some of which contained 500 nM geminin (lanes 3 and 4). Subsequently, NPE was added at 19°C, and after 25 min p27^{Kip} was added. After a further 15 min, the chromatin was isolated and exposed to buffer, Rb¹⁻⁴⁰⁰, or Rb¹⁻⁴⁰⁰/MCM7 peptide. Fresh NPE containing buffer, Rb¹⁻⁴⁰⁰ or Rb¹⁻⁴⁰⁰/MCM7 peptide was added, and DNA replication was measured after 45 min.

some unwinding (Figure 2B). After isolation, the chromatin was incubated with buffer or purified anti-Cdc45 IgG. The chromatin was washed and supplemented with fresh NPE lacking antibody but containing [α]³²P-dATP. As shown in the bar graph in Figure 5A, Cdc45 antibody eliminated DNA replication (compare columns 2 and 3), and the inhibitory effect was reversed by pre-incubating the antibody with recombinant Cdc45 protein (column 4). In a control experiment, we found that anti-geminin antibody did not inhibit DNA replication (data not shown). We repeated the same experiment but used NPE^{aph} for the final incubation, and found that Cdc45 antibody blocked RPA hyperloading (Figure 5A, upper panel). The apparent excess of ORC2 present in lane 4 represents the purified his-tagged Cdc45 protein, which sticks nonspecifically to chromatin, and is detected because we blot for ORC2 and Cdc45 simultaneously. The results in Figure 5A argue that Cdc45 plays a crucial role in chromosome unwinding by origin-proximal replication complexes.

To determine whether Cdc45 is required for the elongation phase of DNA replication, we used low-temperature synchronization. DNA replication was allowed to initiate in NPE for

25 min at 19°C, p27^{Kip} was added, and after a further 15 min, chromatin was isolated, or incubated with buffer, Cdc45 antibody, or Cdc45 antibody pre-incubated with Cdc45 protein. After incubation, excess supernatant was removed and replaced with fresh NPE^{Kip} to measure DNA replication (Figure 5B, bar graph), or NPE^{Kip+aph} to measure helicase-uncoupled hyperunwinding (Figure 5B, upper panel). Whereas the buffer control exhibited significant additional DNA replication and hyperunwinding upon transfer of chromatin to the second NPE (Figure 5B, compare conditions 1 and 2), the reaction containing Cdc45 antibody did not (condition 3), and preincubation of Cdc45 antibody with Cdc45 protein reversed the inhibition (condition 4). These results argue that Cdc45 is required for chromosome unwinding during elongation.

It is noteworthy that, in Figure 5, the Cdc45 antibody completely inhibited DNA replication and unwinding, even though no Cdc45 antibody was added with the second NPE, which contains high concentrations of Cdc45 protein. This result suggests that the Cdc45 initially loaded onto chromatin (either in actD or at 19°C) has a very slow off-rate. However, it was possible that the antibody locked the replication complex into a conformation in which Cdc45 was not able to dissociate. To further explore the Cdc45 off-rate, we loaded Cdc45 onto chromatin in NPE^{actD}, and then transferred the chromatin to fresh NPE that was Cdc45 depleted. DNA replication in the Cdc45-depleted NPE was reduced only about 50% compared to the mock-depleted extract (Figure 6, bar graph, columns 1 and 2). A similarly modest effect was observed on RPA hyperloading and Cdc45 binding itself (Figure 6, lower panel). Importantly, the depleted NPE was unable to support DNA replication, Cdc45 loading, or RPA hyperloading of naive chromatin (Figure 6, compare conditions 3 and 4), demonstrating that it lacked functional levels of Cdc45. We conclude that chromatin-bound Cdc45 has a slow off-rate and that extensive DNA replication can occur without Cdc45 dissociation. Interestingly, the fact that replication is reduced by 50% when no free Cdc45 is present implies that free Cdc45 can normally reload onto replication complexes that have lost the protein, as seen in yeast (Tercero *et al*, 2000).



Discussion

Previous results using *Xenopus* egg extracts showed that the MCM complex is required for pre-RC and pre-IC assembly (Cdc45 loading) in metazoans, but it was unclear whether MCM participates in subsequent steps of DNA replication

Figure 5 Cdc45 is required for chromosome unwinding during elongation. (A) Cdc45 is required for chromosome unwinding near origins. After incubation in HSS followed by NPE^{actD}, chromatin was isolated and stopped (lane 1), or isolated and exposed to buffer (lane 2), 0.4 µg/µl Cdc45 IgG (lane 3), or Cdc45 IgG that had been preincubated with 90 ng/µl purified His-tagged Cdc45 protein (lane 4). After 30 min, the chromatin was washed, and fresh NPE was added, which contained aphidicolin to measure chromatin loading of RPA or [α -³²P]dATP to measure DNA replication. (B) The same low-temperature synchronization approach used in Figure 3C was used, except that inhibition was carried out using Cdc45 antibodies as in (A), and DNA replication (bar graph), or chromatin loading of MCM7, ORC2, Cdc45, and RPA in the presence of aphidicolin (upper panel) was measured.

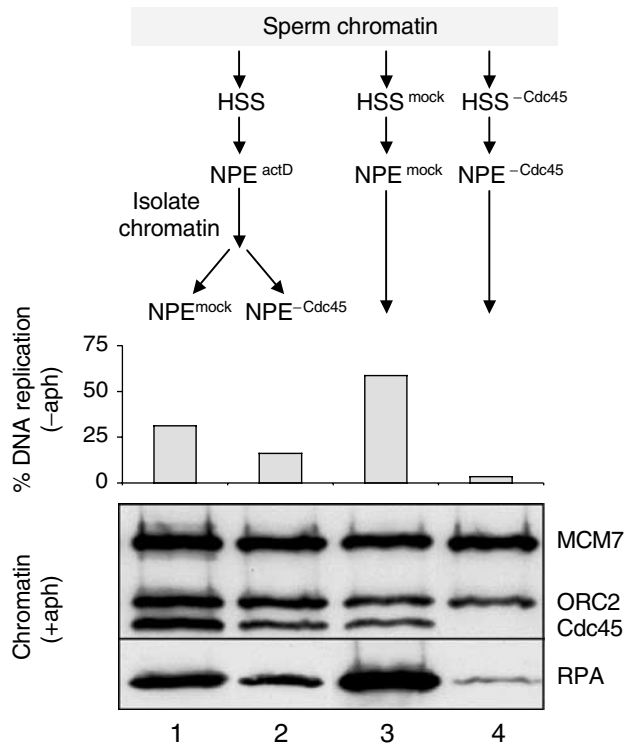


Figure 6 Cdc45 has a slow off-rate from chromatin. Sperm chromatin containing the activated helicase was generated and purified as in Figure 2C. Subsequently, it was incubated with mock-depleted (lane 1) or Cdc45-depleted NPE (lane 2). In the same experiment, sperm chromatin was incubated with mock-depleted HSS and NPE (lane 3) or Cdc45-depleted HSS and NPE (lane 4). At 45 min after the final NPE addition, DNA replication and chromatin loading in the presence of aphidicolin were measured.

including origin unwinding and elongation. In this report, we used Rb¹⁻⁴⁰⁰ to target the MCM7 protein after pre-RC assembly. Rb¹⁻⁴⁰⁰ inhibited DNA replication when it was added after pre-RC formation, immediately after origin unwinding, and after 30% of the input DNA had replicated. These data indicate that MCM7 is required for elongation in metazoans. We also examined the effects of Rb¹⁻⁴⁰⁰ on DNA unwinding by uncoupled DNA helicases. First, we showed that Rb¹⁻⁴⁰⁰ added immediately after pre-RC formation allowed Cdc45 loading but blocked aphidicolin-induced hyperunwinding, suggesting that MCM7 is required for the activity of the helicase that unwinds the origin. To rule out that Rb¹⁻⁴⁰⁰ inhibits an unknown helicase activation step, Rb¹⁻⁴⁰⁰ was added after the helicase had been activated in the presence of actD. The hyperunwinding observed when replication complexes are transferred from NPE^{actD} to NPE^{aph} likely reflects uncoupling of the helicase that unwound the origin. Based on the inhibition of this helicase by Rb¹⁻⁴⁰⁰, we infer that MCM7 is part of the molecular machine that unwinds origins. Finally, replication complexes were allowed to synthesize 30% of the DNA before transfer of the chromatin to the extract containing aphidicolin to induce hyperunwinding. Inhibition of hyperunwinding in this setting by Rb¹⁻⁴⁰⁰ argues that MCM7 is required for unwinding of DNA along the entire replicon.

Although the simplest interpretation of the inhibitory effects of Rb¹⁻⁴⁰⁰ is that it targets MCM7 and thereby prevents chromosome unwinding, alternative explanations exist. First,

it is possible that Rb¹⁻⁴⁰⁰ binds to MCM7 and then disrupts another protein at the replication fork, which is the actual helicase. Given the great stability with which the eukaryotic replicative DNA helicase is expected to bind to DNA, this scenario appears unlikely. Second, the binding of Rb¹⁻⁴⁰⁰ to the multitude of chromatin-bound MCM2-7 complexes might create a repressive chromatin structure that cannot be traversed by the replicative DNA helicase. However, we showed that chromatin containing much lower levels of MCM2-7 was still susceptible to inhibition by Rb¹⁻⁴⁰⁰. Third, Rb might cause inhibition by targeting a protein other than MCM7. However, such a factor would have to closely resemble MCM7, since the inhibitory effect of Rb¹⁻⁴⁰⁰ is completely reversed by an MCM7 peptide. In addition, we showed that loading of Rb¹⁻⁴⁰⁰ onto chromatin (the most likely site of the target) was dependent on chromatin-bound MCM2-7 and sensitive to MCM7 peptide. Finally, the inhibition of DNA replication and chromosome unwinding by Rb¹⁻⁴⁰⁰ was reversed by co-addition of Cdk4/Cyclin D (M Pacek, Andrew Gladden, Alan Diehl, and JC Walter, unpublished results). Since the interaction between MCM7 and Rb is also sensitive to Cdk4/Cyclin D (Gladden and Diehl, 2003), this finding provides further evidence that Rb¹⁻⁴⁰⁰ specifically targets MCM7 in extracts.

Our results provide a new class of support for the idea that the MCM complex is the eukaryotic replicative DNA helicase. Previous work showed that: (1) MCM2-7 travels with and is required for progression of the replication fork in yeast (Aparicio *et al*, 1997; Labib *et al*, 2000); (2) MCM complexes are structurally similar to replicative DNA helicases (Chong *et al*, 2000; Fletcher *et al*, 2003); (3) MCM4/6/7 subassemblies exhibit helicase activity *in vitro* (You *et al*, 1999; Lee and Hurwitz, 2000; Kaplan *et al*, 2003). We now show that MCM7 is directly required for DNA unwinding in the context of replication-competent chromatin. It is important to note that our data do not address whether an MCM2-7 holocomplex, an MCM4/6/7 subcomplex, or some other MCM7-containing complex unwinds chromosomes. However, given that at least five MCM subunits are required for elongation in *Saccharomyces cerevisiae* (Labib *et al*, 2000), the simplest interpretation of our results is that the MCM2-7 holocomplex is the replicative DNA helicase and that MCM7 is an essential component of this enzyme.

The conclusion that MCM2-7 is required for elongation in vertebrates is noteworthy because previous immunofluorescence (IF) studies failed to place the MCM2-7 complex at sites of ongoing DNA replication (Todorov *et al*, 1994; Krude *et al*, 1996; Romanowski *et al*, 1996). Previously, we and others showed that, in *Xenopus* egg extracts, a large number of MCM2-7 complexes are widely distributed on chromatin, and that only some of these are activated for DNA replication (Edwards *et al*, 2002; Harvey and Newport, 2003). Evidence for a similar phenomenon exists in mammalian cells (see Discussion in Edwards *et al*, 2002). We therefore postulated that the MCM2-7 complexes present at the replication fork are invisible by IF due to the large number of latent complexes present elsewhere. Our current demonstration that MCM7 is required for replication elongation in metazoans supports this interpretation since it implies that there must be MCM complexes present at the replication fork.

Like MCM2-7, Cdc45 has been shown to travel with and be required for replication fork progression in yeast (Aparicio

et al, 1997; Tercero *et al*, 2000), and Cdc45 and MCM2-7 are found in the same complex on chromatin (Zou and Stillman, 1998; Mimura *et al*, 2000). When MCM2-7 is recovered from chromatin that was assembled in the absence of Cdc45 in *Xenopus* egg extracts, MCM2-7-associated helicase activity is reduced (Masuda *et al*, 2003). These data are consistent with Cdc45 playing a role in pre-IC formation, but do not demonstrate an ongoing requirement for Cdc45 in helicase activity. We added Cdc45 antibody to replication complexes arrested immediately after origin unwinding or during elongation. In both cases, Cdc45 antibody blocked further DNA replication, demonstrating a requirement for Cdc45 in elongation. Importantly, Cdc45 antibodies also blocked hyperunwinding in the presence of aphidicolin, indicating that this protein is required for the activity of the replicative DNA helicase. Since Cdc45 itself has no recognizable ATPase motifs, the simplest interpretation of these results is that it functions as a helicase co-factor. Interestingly, once Cdc45 has been loaded, transfer of chromatin into Cdc45-deficient extract caused only an ~50% reduction in DNA replication, suggesting that Cdc45 does not rapidly cycle on and off the chromatin.

Although we used Rb¹⁻⁴⁰⁰ primarily as a tool to target the MCM2-7 complex, it is interesting to consider the *in vivo* role of this interaction. The canonical function of Rb is to bind to E2F and prevent transcription of S-phase-specific genes (Sherr, 1996). Activation of Cdk4/Cyclin D by growth factors leads to phosphorylation and inactivation of Rb such that cells can proceed into the S phase, and Rb remains phosphorylated for the remainder of the cell cycle. Similar to the Rb-E2F interaction, the Rb-MCM7 interaction is negatively regulated by Cdk4/Cyclin D (Gladden and Diehl, 2003), and this protein kinase abolishes the inhibitory effects of Rb on DNA replication and chromosome unwinding (M Pacek, Andrew Gladden, Alan Diehl, and JC Walter, unpublished observations). Together, these observations indicate that Rb could restrain S phase by an additional mechanism that involves inhibition of the replicative DNA helicase. Interestingly, Cyclin D is destroyed and Rb is dephosphorylated when cells are exposed to DNA-damaging agents (Lan *et al*, 2002), and recent data indicate that, under these conditions, Rb binds to origins of DNA replication (Avni *et al*, 2003). Thus, in the G1 phase, and in response to cell cycle checkpoints, the hypophosphorylated form of Rb might inhibit the helicase activity of the MCM2-7 complex and thereby prevent unscheduled DNA replication.

Materials and methods

DNA replication, chromatin binding, and DNA topology assays

For DNA replication assays, HSS was incubated with sperm chromatin (10 000 sperm/ μ l) for 30 min at 22°C, followed by

addition of 2 volumes NPE containing trace amounts of [α -³²P]dATP (Walter *et al*, 1998). Incorporation of [α -³²P]dATP was measured using agarose gel electrophoresis (Dasso and Newport, 1990), and the percentage of input DNA replicated was calculated by determining the fraction of free label consumed, assuming a 50 μ M endogenous pool of dATP (Blow and Laskey, 1986).

For chromatin-binding assays, the extract containing sperm chromatin (up to 10 μ l) was diluted with 60 μ l of cold ELB/TX-100 buffer (0.25 M sucrose, 2.5 mM MgCl₂, 50 mM KCl, 10 mM HEPES pH 7.7 and 0.2% Triton X-100), layered onto a 180 μ l sucrose cushion (ELB containing 0.5 M sucrose) in Beckman 5 \times 44 mm microcentrifuge tubes, and isolated by centrifugation for 25 s at 12 000g (Walter *et al*, 1998). After washing twice with ELB, SDS sample buffer was added.

For the chromatin transfer experiments in Figures 2C, 5A, and 6, sperm chromatin was incubated in 2.5 μ l of HSS, followed by 5 μ l of NPE containing 10 μ M actinomycin D (NPE^{actD}), and then isolated as described above. The chromatin was incubated with the appropriate inhibitor for 30 min at 22°C. Subsequently, excess liquid was aspirated, and the chromatin was washed three times in ELB. Next, 5 μ l of fresh NPE that contained either 50 μ M aphidicolin to measure chromosome unwinding, or trace amounts of [α -³²P]dATP to measure DNA replication was added. After 45 min, chromatin loading or DNA replication was measured. The low-temperature synchronization experiment was performed in the same manner, except that, after incubation of sperm chromatin in HSS, NPE lacking actD was added on ice and the reaction was incubated at 19°C for 25 min, followed by the addition of 2 μ M p27^{Kip} for another 15 min. The chromatin was then isolated and further processed as described above. The DNA topology assay was carried out as described (Walter and Newport, 2000).

Protein purification and immunological techniques

The 400 amino-acid GST-tagged N-terminal fragment of Rb and the 137 amino-acid MBP-tagged C-terminal MCM7 peptide were purified as described (Sterner *et al*, 1998). his-Cdc45 was purified as described (Walter and Newport, 2000). Western blotting was carried out using an RPA polyclonal rabbit antibody (Walter and Newport, 2000) or a mixture of ORC2 (Walter and Newport, 1997), MCM7 (Walter and Newport, 2000), and Cdc45 (Walter and Newport, 2000) polyclonal rabbit antibodies. The 34 kDa subunit of RPA is shown. Immunodepletion of Cdc45 protein was carried out as described (Walter and Newport, 2000). The Cdc45 IgG used to inhibit Cdc45 function was generated by acidic elution from rSepharose A FastFlow resin. The concentration of the eluted total IgG preparation was ~2 mg/ml.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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