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MCM2–7 Complexes Bind Chromatin in a Distributed Pattern Surrounding the Origin Recognition Complex in *Xenopus* Egg Extracts*

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The MCM2–7 complex is believed to function as the eukaryotic replicative DNA helicase. It is recruited to chromatin by the origin recognition complex (ORC), Cdc6, and Cdt1, and it is activated at the G_1/S transition by Cdc45 and the protein kinases Cdc7 and Cdk2. Paradoxically, the number of chromatin-bound MCM complexes greatly exceeds the number of bound ORC complexes. To understand how the high MCM2–7:ORC ratio comes about, we examined the binding of these proteins to immobilized linear DNA fragments in *Xenopus* egg extracts. The minimum length of DNA required to recruit ORC and MCM2–7 was ~80 bp, and the MCM2–7:ORC ratio on this fragment was ~1:1. With longer DNA fragments, the MCM2–7:ORC ratio increased dramatically, indicating that MCM complexes normally become distributed over a large region of DNA surrounding ORC. Only a small subset of the chromatin-bound MCM2–7 complexes recruited Cdc45 at the onset of DNA replication, and unlike Cdc45, MCM2–7 was not limiting for DNA replication. However, all the chromatin-bound MCM complexes may be functional, because they were phosphorylated in a Cdc7-dependent fashion, and because they could be induced to support Cdk2-dependent Cdc45 loading. The data suggest that in *Xenopus* egg extracts, origins of replication contain multiple, distributed, initiation-competent MCM2–7 complexes.

In eukaryotic organisms, DNA replication initiates at many sites (1). In *Saccharomyces cerevisiae*, DNA replication initiates every ~40 kb at autonomously replicating sequences that recruit the origin recognition complex (ORC),[1] the six-subunit initiator protein. In metazoans, initiation sites are less rigidly defined. In embryonic cells of *Xenopus laevis*, DNA replication initiates once every ~10 kb without sequence specificity (2). In somatic cells, initiation events are less frequent, occurring once every ~150 kb, and recent evidence indicates that initiations are controlled by genetic elements (1). At some loci, replication initiates at a precise location, whereas at other loci, initiation events are distributed throughout zones spanning up to 50 kb.

The mechanism of DNA replication initiation is highly conserved among eukaryotic organisms (3, 4). A representative model system is *Xenopus* egg extracts (2, 4) where two factors, Cdc6 and Cdt1, bind to sites on chromatin that are marked by ORC. Subsequently, the hexameric MCM2–7 complex binds to the ORC-Cdc6-Cdt1 complex to establish the pre-replication complex (pre-RC). At the onset of DNA replication, the pre-RC is activated by the sequential action of the protein kinases Cdc7/Dbf4 and Cdk2/cyclin E (Cdk) (5, 6). Genetic and biochemical experiments suggest that the function of Cdc7/Dbf4 is the phosphorylation of the MCM2–7 complex (7). After MCM phosphorylation by Cdc7/Dbf4, Cdk2/cyclin E stimulates the association of Cdc45 with the pre-RC, likely via a direct interaction with the MCM2–7 complex (8, 9). The binding of Cdc45 coincides with activation of a chromatin-bound helicase that unwinds the DNA, allowing binding of the single-stranded DNA-binding protein, RPA, and DNA polymerase α/primase (10, 11). During replication, the MCM complex is displaced from chromatin, and its re-association is inhibited until cells pass through mitosis. In *Xenopus* egg extracts, re-replication is prevented by at least two inhibitors that block the re-loading of the MCM complex onto origins: geminin (12), which negatively regulates the MCM complex loading factor Cdt1 (13, 14); and Cdk2/cyclin E (15), which appears to inhibit MCM2–7 loading by multiple mechanisms, one of which involves nuclear export of Cdc6 (16).

Biochemical and genetic experiments provide strong support for the idea that the MCM2–7 complex functions as the replicative DNA helicase (reviewed in Ref. 17). However, several observations are not easily reconciled with this model. Immunofluorescence studies in *Xenopus* egg extracts and mammalian cells show that the majority of MCM complexes do not co-localize with sites of DNA synthesis (18–21). Rather, the MCM complex appears to bind to unreplicated chromatin close to the site of synthesis (19). Moreover, chromatin immunoprecipitation experiments suggest that MCM and ORC do not co-localize on DNA fragments as large as 500–1000 bp (22). Another puzzling observation concerns the number of chromatin-bound ORC and MCM complexes. In *Xenopus* egg extracts, the number of ORC complexes closely matches the number of initiation events, whereas the number of MCM complexes exceeds the number of initiation events by a factor of ~10–40 (23, 24). In *S. cerevisiae* and humans, the number of chromatin-bound MCM complexes also greatly exceeds the number of origins (25–29). Together these findings appear to give rise to an "MCM paradox" in which multiple DNA helicases are present in nuclei at sites that do not coincide with replication forks

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[1] The abbreviations used are: ORC, the origin recognition complex; pre-RC, pre-replication complex; NPE, nucleoplasmic extract; RPA, replication protein A.

(18). The MCM paradox could be resolved if MCM complexes were bound in a distributed pattern along eukaryotic chromosomes, as proposed previously (19, 30). In this paper, we use *Xenopus* egg extracts to provide direct evidence for this idea. Moreover, our data argue that whereas most chromatin-bound MCM complexes are competent for replication initiation, only a small subset is normally used during S phase.

EXPERIMENTAL PROCEDURES

Immobilization, Restriction, and Analysis of Linear DNA Fragments on Magnetic Beads—To generate biotinylated DNA fragments, PCR was carried out with pairs of oligonucleotide primers 15–28 bp in length (exact sequences available upon request), one of which contained a biotin molecule attached to the 5′-nucleotide via a 15-atom linker arm (Operon Technologies). PCR products were generated using as a template pRS415 (6021 bp (31)) that was linearized with *Sca*I at position 4998. To generate a 6017-bp ("6 kb") DNA fragment, PCR primers starting at positions 4998 (biotinylated) and 5003 were used. To generate the 1006-bp ("1 kb") DNA fragment, PCR primers starting at positions 210 and 1215 (biotinylated) were used. To generate a 251-bp ("0.25 kb") DNA fragment spanning the polylinker, PCR primers starting at positions 3041 and 3291 (biotinylated) were used. To generate a 251-bp (0.25 kb) DNA fragment containing an *Eco*RI site near the biotinylated end, PCR primers starting at positions 1112 and 1362 (biotinylated) were used. To generate the 2957-bp ("3 kb") DNA fragment used in Fig. 1, we used as a DNA template *Sca*I-digested pBS KS(−) and the same primers used to generate the 6-kb DNA fragment above.

PCR products were purified twice using a PCR purification kit (Qiagen) and then coupled to streptavidin-coated magnetic beads using the Kilobasebinder kit (Dynal). Coupling reactions contained 5 μg/μl beads and ~50 (6-kb fragments), 20 (3-kb DNA fragments), 50 (1-kb DNA fragments), or 25 ng/μl DNA (0.25-kb DNA fragments). After coupling according to the manufacturer's instructions, beads were resuspended in ELB (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes, pH 7.7) at 10 μg/μl and digested with 0.1 mg/ml P1 nuclease (Roche Molecular Biochemicals) for 2 min at 37 °C to destroy single-stranded DNA. Subsequently, beads were washed three times in 10 mM Tris, pH 8, 20 mM EDTA and stored at 4 °C for up to several weeks. Coupling efficiencies ranged from ~60 (0.25-kb DNA fragments) to ~20% (6-kb DNA fragments).

To digest immobilized DNA fragments, beads were resuspended in the appropriate restriction enzyme buffer (New England Biolabs) at 5 μg/μl. 0.5–1 units of enzyme/5 μg of beads were added, and digestion was carried out for 60 min on a rotating wheel at 37 °C. Beads were washed twice in 2 volumes of 10 mM Tris, pH 8, 20 mM EDTA, and resuspended in the same buffer at 10 μg/μl. To assess the extent of digestion, 50 μg of beads were resuspended in TBE loading dye containing 10 mM EDTA and boiled for 3 min. After snap-cooling, beads were centrifuged for 1 min at 16,000 × *g*. 1 μl of fresh 20 mg/ml proteinase K (Roche Molecular Biochemicals) was added; the beads were resuspended, and the mixture was digested at 37 °C for 30 min. The sample was boiled again for 3 min, snap-cooled, and centrifuged, and the entire sample (including the beads) was loaded onto a 1% agarose gel (2% for DNA fragments ≤250 bp). The DNA was stained with SYBR gold (Molecular Probes) according to the manufacturer's instructions. Digestion was always verified to be ~100% complete.

To generate a series of DNA fragments 6017 (6 kb), 2824 (3 kb), 955 (1 kb), and 358 ("0.35 kb") bp in length, the immobilized 6-kb DNA fragment was digested with buffer or *Hpa*I, *Alw*NI, or *Bgl*I, respectively. To generate immobilized DNA fragments 251, 154, 120, 94, 82, 67, and 54 bp in length, the 251-bp DNA fragment spanning the polylinker of pRS415 was digested with buffer, *Apa*I, *Eco*RV, *Bam*HI, *Xba*I, *Sac*II, or *Alu*I, respectively. To generate immobilized DNA fragments 1006 (1 kb) and 95 ("0.1 kb") bp in length, the immobilized 1006-bp DNA fragment was digested with buffer or *Eco*RI. The length of the DNA fragments refers to the amount of duplex DNA.

Chromatin Binding—Unless stated otherwise, chromatin binding experiments were performed as follows. 100 μg of DNA beads were incubated with 10 μl of *Xenopus* egg cytosol containing 3 μg/ml nocodozole and an ATP regeneration system (32). After rotating the beads for 20 min at room temperature (21–23 °C), the extract was diluted with 60 μl of ELB supplemented with 50 mM KCl and 0.2% Triton X-100 and layered onto a 180-μl sucrose cushion (ELB + 0.25 M sucrose) in Beckman 5 × 44-mm Microfuge tubes. Samples were centrifuged horizontally for 25 s at 16,000 × *g*. The supernatant was removed and replaced with 200 μl of cold dilution buffer, and the sample was centrifuged in the same way. After removal of the supernatant, the beads were resuspended in SDS-PAGE sample buffer. In some cases, egg cytosol was preincubated for 5 min with 150 nM human geminin (14). For chromatin binding in NPE, beads were collected with a magnet after the incubation in egg cytosol; 80% of the supernatant was removed, and it was replaced with 1 volume of NPE. After incubation in NPE for 20 min, chromatin was purified as described above. When binding to immobilized DNA fragments of different sizes was compared, it involved DNAs that had been derived from the same parent fragment by digestion. The exception is in Fig. 4*A*, where the 100-bp fragment was derived by digestion from a 250-bp parent fragment. In Fig. 4*C*, the 100-bp fragment was derived from the 1-kb fragment, but the 6-kb fragment was coupled to beads separately. The protocol for chromatin binding on demembranated sperm was the same as for DNA beads, except that the sperm was diluted with ELB containing 0.2% Triton X-100 before application to the cushion, and it was washed with ELB. DNA replication assays, egg cytosol preparation, and NPE preparation were carried out as described (32).

Immunological Techniques—Western blotting was carried out using ORC2 (24), MCM3 (15), MCM7 (11), Cdc7 (11), Cdc45 (11), and RPA (11) rabbit antibodies. In all cases, the 34-kDa subunit of RPA is shown. Sometimes Western blots were probed with a mixture of Cdc45 and ORC2 antibodies. Cdc6 antibodies were raised in rabbits against his-Cdc6 expressed in insect cells. Immunodepletions of MCM7 and CDC45 were carried out as described (11), except that Cdc45 was removed with 2 rounds of depletion. For quantitative Western blotting, protein concentrations of purified his-ORC2 (33), his-Cdc45 (8), and MCM3 (34) were estimated by Coomassie Blue staining and densitometry using bovine serum albumin standards.

RESULTS

Assembly of Functional Pre-replication Complexes on Immobilized Linear DNA Fragments—We employ a soluble cell-free system derived from *Xenopus* eggs to study eukaryotic DNA replication (32). Demembranated sperm chromatin or plasmid DNA is incubated with a cytosolic egg extract to assemble pre-RCs containing ORC, Cdc6, Cdt1, and the MCM complex. Subsequently, a highly concentrated nucleoplasmic extract (NPE) is added. Shortly after NPE addition, pre-RCs initiate replication and a single complete round of DNA replication occurs with *in vivo* kinetics.

To understand the spatial relationship on DNA between ORC and the MCM complex, we developed a system in which binding of these factors could be measured on immobilized linear DNA fragments. A 3-kb DNA fragment biotinylated at one end was generated by PCR and bound to streptavidin-coated magnetic beads. The immobilized DNA was incubated with *Xenopus* egg cytosol for 20 min, a treatment that did not lead to detectable degradation or ligation of the DNA (data not shown). The beads were recovered and washed gently, and bound proteins were analyzed by Western blotting. Both ORC2 and MCM3 were detected on beads containing the PCR product but not on an equivalent quantity of beads lacking DNA (Fig. 1, compare *lanes 1* and *2*). Geminin blocked the association of MCM3 with beads (Fig. 1, compare *lanes 3* and *4*), demonstrating that loading was Cdt1-dependent (14). The characteristic increase in ORC2 binding seen in the presence of geminin on sperm chromatin (12) was also seen on immobilized linear DNA templates (Fig. 1, compare *lanes 3* and *4*). As expected, immunodepletion of Cdc6, ORC2, or Cdt1 from extracts also completely abolished MCM3 binding to DNA beads (data not shown). Together, these observations show that pre-RCs are assembled via physiological mechanisms on immobilized linear DNA templates.

To test whether immobilized pre-RCs are functional, they were mixed with NPE. Addition of NPE led to the binding of Cdc45 (Fig. 1, *lane 5*), and binding was blocked by the Cdk2-inhibitor, $p27^{Kip}$ (Fig. 1, *lane 6*), as expected because Cdc45 binding requires Cdk2 (8, 9). Therefore, pre-RCs formed on immobilized linear DNA templates can be transformed into a pre-initiation complex. Despite loading of Cdc45, immobilized DNA templates did not undergo efficient DNA replication (data

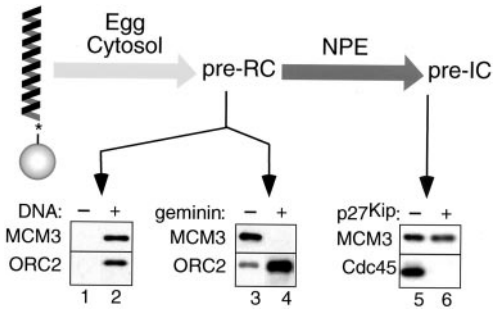

FIG. 1. **Pre-RCs are formed and activated on immobilized linear DNA fragments.** Equivalent amounts of magnetic beads (*lane 1*) or magnetic beads coupled to a 3-kb DNA fragment of pBluescript (*lanes 2–6*) were incubated with egg cytosol for 20 min, isolated, washed, and analyzed by Western blotting. In *lane 4*, the egg cytosol contained geminin. In *lanes 5* and *6*, the incubation in egg cytosol was followed by 15 min of incubation in aphidicolin-supplemented NPE that contained (*lane 6*) or lacked (*lane 5*) 1 μM $p27^{Kip}$. Western blots were probed with MCM3 (*all panels*), ORC2 (*left* and *center panels*), or Cdc45 (*right panel*).

not shown). However, PCR products that were not coupled to beads also failed to replicate (data not shown). Addition of caffeine did not rescue replication of linear DNA fragments (data not shown), indicating that the failure to replicate was not because of a checkpoint response induced by the presence of double-stranded DNA ends (35, 36). Thus, whereas linear molecules do not replicate in this system, when immobilized, they represent a powerful approach to investigate the mechanism of pre-RC and pre-initiation complex assembly.

Assembly of a Pre-RC Requires 82 bp—If MCM is distributed along the length of chromosomes, a long fragment of DNA should be required to achieve the high ratio of MCM:ORC observed on sperm chromatin (23, 24). On the other hand, if MCM complexes bind chromatin in the vicinity of ORC, the minimum length of DNA required to assemble ORC should also be sufficient to recruit many MCM complexes. To distinguish between these possibilities, we first determined the minimum amount of DNA required to recruit ORC and MCM complexes in *Xenopus* extracts. A 250-bp PCR product spanning a multiple cloning site was coupled to beads. Equal quantities of these beads were then digested with different restriction enzymes to generate immobilized DNA fragments of 251, 154, 120, 94, and 67 bp (Fig. 2*A*). As seen by the absence of the parent DNA fragment in *lanes 2–5* in Fig. 2*A*, digestion by the restriction enzymes was complete. By digesting the DNA after immobilization, potential differences in coupling efficiency between DNAs that differ in length was eliminated, and we were able to examine binding to equimolar amounts of DNAs.

When equal quantities of the digested beads were incubated with egg cytosol, ORC2 and MCM3 each bound to the 251-, 154-, 120-, and 94-bp DNA fragments (Fig. 2*B*, *lanes 1–4*), but very little binding was seen on the 67-bp DNA fragment (Fig. 2*B*, *lane 5*). Interestingly, the amount of ORC2 bound to all fragments longer than 94 bp was essentially the same, whereas the amount of MCM3 bound increased substantially (Fig. 2*B*, *lanes 1–4*). Addition of geminin to the egg cytosol abolished binding of MCM3 to all the DNA fragments (Fig. 2*C*, *lanes 6–9*). We next used quantitative Western blotting to examine the ratio of MCM3 and ORC2 on equal amounts of 251- and 94-bp DNA beads (Fig. 2*D*). This was done by analyzing DNA-bound material (Fig. 2*D*, *lanes 1* and *2*) alongside a dilution series of a 1:1 molar ratio of purified MCM3:ORC2 (Fig. 2*D*, *lanes 3–6*). The ratio of MCM3:ORC2 on the 94-bp DNA fragment was very close to 1:1 (Fig. 2*D*, compare *lanes 1* and *5*), whereas it was higher on the 250-bp DNA fragment (Fig. 2*D*, *lane 2*). Therefore, on DNA fragments of 120 bp or less, only ~1 MCM complex is

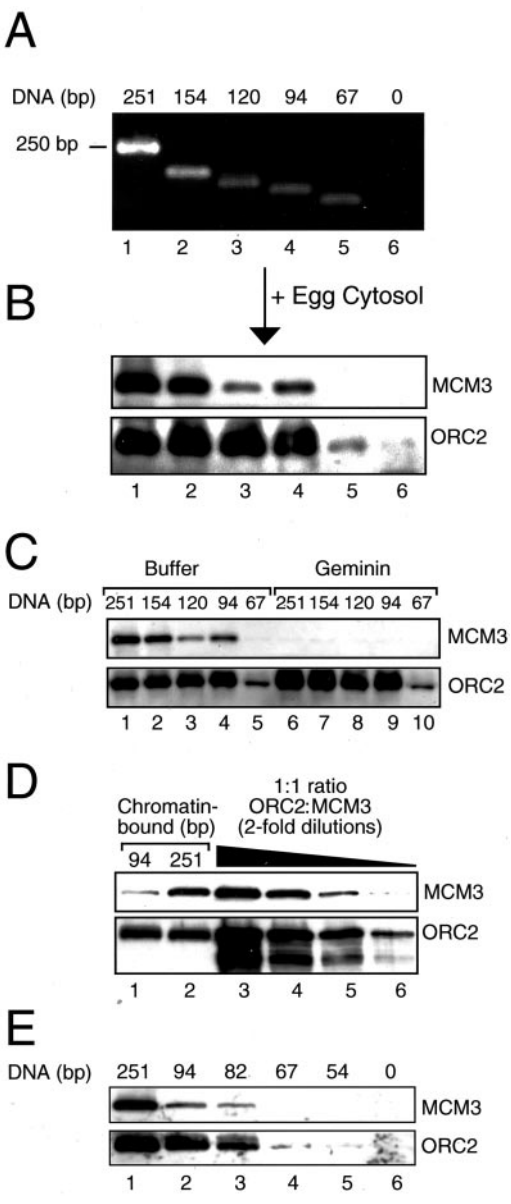

FIG. 2. **ORC and MCM are efficiently recruited to an 82-bp DNA fragment.** *A* and *B*, a 251-bp PCR product spanning the pBluescript polylinker was coupled to beads. The beads were divided into equal-sized pools and mock-digested or digested with different enzymes to generate 251 (*lane 1*), 154 (*lane 2*), 120 (*lane 3*), 94 (*lane 4*), and 67 (*lane 5*)-bp DNA fragments. *Lane 6* contained beads lacking DNA. After digestion, DNA attached to beads was analyzed on a 2% agarose gel (*A*), or equal quantities of the various beads were incubated with egg cytosol and the attached proteins analyzed by Western blotting (*B*). *C*, a similar set of DNA beads as those used in *A* were incubated with egg cytosol containing buffer (*lanes 1–5*) or geminin (*lanes 6–10*), and the bound proteins were analyzed by Western blotting. *D*, proteins bound to equivalent amounts of 94-bp beads (*lane 1*) and 251-bp beads (*lane 2*) were analyzed by Western blotting alongside 2-fold dilutions of a 1:1 molar ratio of purified MCM3 and ORC2 (*lanes 3–6*). The amount of ORC2 and MCM3 in *lane 3* is 16 and 24.6 ng, respectively. *E*, binding of proteins to digested DNA beads as in *B* but with the addition of 82- (*lane 3*) and 54-bp (*lane 5*) fragments. As in *B*, all lanes contained equivalent amounts of beads that were all derived by digestion from the 251-bp parental DNA fragment.

recruited per ORC complex. On fragments of 154 bp and greater, it appears that more than one MCM complex is recruited (see below). To uncover possible differences in the minimum length of DNA required to load ORC and MCM2–7, we generated another fragment size of 82 bp. Both ORC2 and MCM3 loaded onto the 82-bp fragment but not onto an equivalent amount of the 67-bp

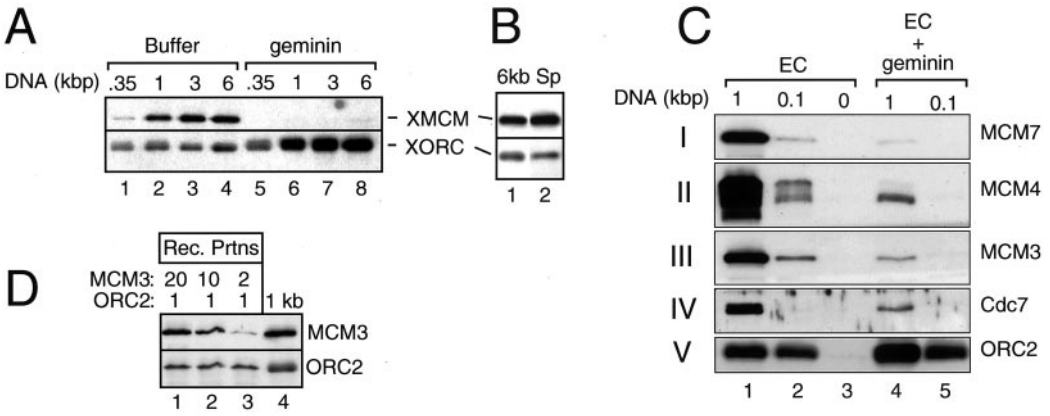

FIG. 3. **MCM complex binding is proportional to the length of DNA fragments attached to beads, whereas ORC binding is independent of DNA fragment length.** *A*, a 6-kb PCR product was coupled to magnetic beads and digested to generate 3-, 1-, and 0.35-kb DNA fragments. Binding of MCM3 and ORC2 to equal amounts of digested beads was examined in egg cytosol in the presence (*lanes 5–8*) and absence (*lanes 1–4*) of geminin. *B*, an immobilized 6-kb DNA fragment (*lane 1*) or sperm (*lane 2*) was incubated in egg cytosol, and binding of MCM3 and ORC2 was examined. *C*, a 1-kb PCR product was attached to beads and digested to generate a 100-bp DNA fragment. Equivalent amounts of the 1-kb DNA beads (*lanes 1* and *4*), 100-bp DNA beads (*lanes 2* and *5*), and beads lacking DNA (*lane 3*) were incubated in egg cytosol (*EC*) containing (*lanes 4* and *5*) or lacking (*lanes 1–3*) geminin, and the attached proteins were analyzed by Western blotting. *D*, proteins bound to an immobilized 1-kb DNA fragment (*lane 4*) were analyzed on a Western blot alongside known quantities of purified MCM3 and ORC2. *Lanes 1–3*, 0.5 ng of ORC2; *lane 1*, 15.3 ng of MCM3; *lane 2*, 7.7 ng of MCM3; *lane 3*, 1.5 ng of MCM3.

fragment (Fig. 2*E*, compare *lanes 3* and *4*). Therefore, ORC was able to recruit efficiently the MCM complex on an 82-bp fragment of DNA, but the MCM2–7:ORC ratio was very low.

MCM Binding to Immobilized Linear DNA Is Proportional to the DNA Fragment Size—We next prepared immobilized DNA fragments that spanned a larger range of sizes. A 6-kb DNA fragment was coupled to beads and digested to generate 3-, 1-, and 0.35-kb DNA fragments. Again, digestion was ~100% efficient (data not shown). When the binding of ORC2 and MCM3 was examined, two interesting results were observed. First, the amount of ORC2 bound did not change significantly on equivalent quantities of DNA beads where the DNA fragment size varied 18-fold from 0.35 to 6 kb (Fig. 3*A*, *lanes 1–4, bottom panel*). This result suggests that the mechanisms that limit ORC binding to once every ~10–15 kb on sperm chromatin (24, 37) and circular plasmids (30) are also operative on linear DNA fragments. Second, in contrast to ORC2, MCM3 loading increased dramatically as the DNA fragment size increased (Fig. 3*A*, *lanes 1–4, top panel*). These observations parallel closely what we found on the 250-bp DNA fragment and its shorter derivatives (Fig. 2*B*). Importantly, the MCM3:ORC2 ratio on the 6-kb DNA fragment was almost as high as that seen on sperm chromatin (Fig. 3*B*, compare *lanes 1* and *2*) where the ratio is typically ~20–40:1 (24) (Fig. 5*A*). This indicates that the mechanism of binding on immobilized DNA fragments is the same as on sperm and that it is not affected by free DNA ends.

Binding of MCM3 to the 6-kb DNA fragment and its derivatives was completely inhibited by geminin (Fig. 3*A*, *lanes 5–8*). Similar to a previous report (12), geminin caused enhanced ORC2 binding, but interestingly, the degree of enhancement was directly proportional to the DNA fragment length (Fig. 3*A*, *lanes 1–8*), suggesting that geminin causes ORC to bind to many sites along the DNA. It is presently not clear whether this is due to inactivation of Cdt1 or to the absence of MCM on the chromatin.

We next sought to determine whether other subunits of the MCM2–7 complex behave similarly to MCM3. To simplify the analysis, we coupled a 1-kb PCR product to beads, and we mock-digested it or digested it to a length of 100 bp. Consistent with the data in Figs. 2*B* and 3*A*, the amount of ORC2 bound per DNA fragment was similar on the 100-bp and 1-kb DNA fragments (Fig. 3*C*, *panel V*, *lanes 1* and *2*), whereas the amount of MCM3 was much greater on the 1-kb fragment than on the 100-bp DNA fragment (Fig. 3*C*, *panel III*, *lanes 1* and *2*). MCM7 (Fig. 3*C*, *panel I*) and MCM4 (Fig. 3*C*, *panel II*), two other subunits of the MCM2–7 complex, behaved similarly to MCM3. Therefore, DNA length-dependent binding appears to be a property of the entire MCM2–7 complex. To determine how many MCM complexes bind per unit length of DNA, we performed quantitative Western blotting on proteins bound to the 1-kb DNA fragment. Densitometry of Fig. 3*D* showed that the ratio of MCM3 to ORC2 on the 1-kb DNA fragment was ~11:1. Assuming that MCM complexes cover the full-length of the DNA fragment, this implies that a single unit of MCM2–7 complex is in contact with ~90 bp of DNA. This is similar to the minimum length of DNA (82 bp) required to load the first MCM2–7 complex (Fig. 2). Together, our observations provide an explanation for the previous finding that the ratio of the MCM2–7 complex to the ORC complex on sperm chromatin is very high (23, 24). The data argue that the MCM complex is delivered to chromatin sites located at a considerable distance from the ORC, resulting in widely distributed binding of many "lateral" MCM complexes on chromatin.

ORC-proximal and ORC-distal MCM Complexes Bind Tightly to Chromatin—We were curious whether ORC-proximal and ORC-distal MCM2–7 complexes interact with DNA via similar mechanisms. To begin to address this question, we compared the stability of MCM2–7 binding on a 6-kb DNA fragment, which contains predominantly ORC-distal MCM complexes, with binding to a 100-bp DNA fragment, which contains only ORC-proximal MCM complexes. MCM complexes loaded onto 6-kb DNA fragments were resistant to 1 M KCl (Fig. 4*A*, *lane 8*). MCM complexes bound to a 100-bp DNA fragment also appeared to be largely resistant to 1 M KCl (Fig. 4*A*, *lane 4*), although in some experiments (data not shown) binding was observed in 0.6 M but not 1 M KCl. In contrast to the MCM complex, ORC was largely displaced from chromatin in 200 mM KCl (Fig. 4*A*, *lanes 2* and *6*), as reported previously (40). We also examined the stability of MCM complexes bound to sperm chromatin and found that they were resistant to 1 M KCl (Fig. 4*B*, *lane 2*). Together, the data show that ORC-distal MCM complexes are at least as salt-resistant as ORC-proximal MCM2–7 complexes, and they argue against the idea that ORC-distal MCM complexes are bound via low affinity interactions with chromatin.

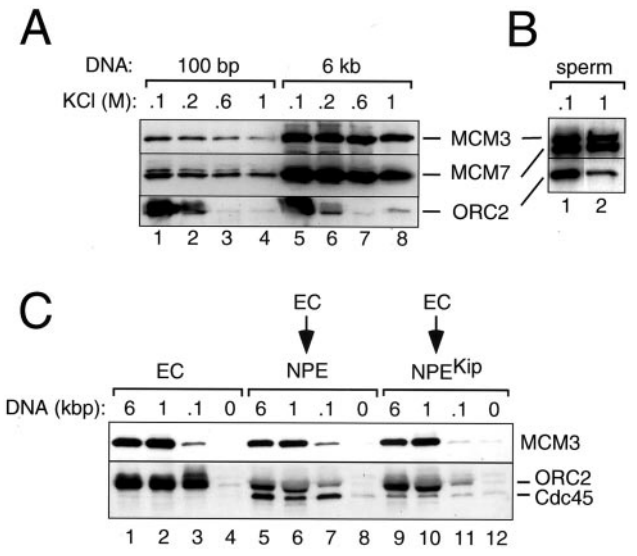

FIG. 4. *A*, lateral MCM complexes are bound tightly to chromatin. 100-bp DNA beads derived by digestion from 251-bp DNA beads (*lanes 1–4*) or 6-kb DNA beads prepared separately (*lanes 5–8*) were incubated with egg cytosol, diluted with buffer containing Triton X-100 and increasing concentrations of salt as indicated, isolated, washed in the same buffer, and probed with MCM3, MCM7, or ORC2 antibodies. *B*, sperm chromatin was incubated with egg cytosol, diluted with buffer containing Triton X-100 and 0.1 M KCl, isolated, washed in buffer containing 0.1 (*lane 1*) or 1 M (*lane 2*) KCl, and probed with ORC2 antibody (*lower panel*) or a mixture of MCM3 and MCM7 antibodies (*upper panel*). *C*, Cdc45 binding is independent of DNA length. Equal quantities of 6-kb DNA beads (*lanes 1*, *5*, and *9*), 1-kb DNA beads (*lanes 2*, *6*, and *10*), or 100-bp DNA beads derived from the 1-kb DNA beads by digestion (*lanes 3*, *7*, and *11*), as well as beads lacking DNA (4, 8, 12), were incubated with egg cytosol (*EC*) (*lanes 1–4*), egg cytosol followed by NPE (*lanes 5–8*), or egg cytosol followed by NPE containing 1 μM $p27^{Kip}$ (*lanes 9–12*). The NPE contained aphidicolin. Chromatin-bound proteins were analyzed by Western blotting using MCM3 antibody (*upper panel*) or a mixture of ORC2 and Cdc45 antibodies (*lower panel*).

Cdc45 Binds to Only a Subset of Chromatin-bound MCM2–7 Complexes—A question of key importance is whether laterally bound MCM complexes, most of which are expected to bind at a considerable distance from ORC, play a role in DNA replication. It was shown previously (38–40) that subsequent to MCM complex loading onto chromatin, ORC and probably Cdc6 and Cdt1 become dispensable for the initiation of DNA replication. This observation suggested that the only function of ORC, Cdc6, and Cdt1 is to deliver the MCM complex to chromatin, and it raises the possibility that laterally bound MCM complexes situated at a distance from ORC may be able to support replication initiation. To address how many chromatin-bound MCM2–7 complexes are activated during initiation, we examined binding of Cdc45 to immobilized DNA fragments upon addition of NPE. We asked whether the loading of Cdc45 onto immobilized linear DNA fragments is proportional to DNA fragment length, as expected if Cdc45 is able to interact with lateral MCM complexes. Unlike MCM2–7, the binding of Cdc45 was independent of the length of the fragment used, being essentially the same on a 1-kb DNA fragment and an equivalent quantity of its 100-bp derivative, as well as a 6-kb DNA fragment prepared separately (Fig. 4*C*, compare *lanes 5–7*). In all cases, binding was Cdk2-dependent (Fig. 4*C*, *lanes 9–11*). As before, the binding of ORC2 was also independent of DNA fragment length (Fig. 4*C*, *lanes 1–3*). These data suggest that only a subset of MCM complexes recruits Cdc45.

As noted above, linear DNA fragments do not replicate efficiently. We therefore asked how many MCM complexes recruit Cdc45 on sperm chromatin, a DNA template that undergoes 100% efficient replication in *Xenopus* egg extracts (32). We determined the ratio of MCM3, ORC2, and Cdc45 on sperm chromatin before and after initiation of DNA replication. Known quantities of purified his-Cdc45 (Fig. 5*A*, *lanes 9–12*) or a mixture of purified his-ORC2 and purified MCM3 (Fig. 5*A*, *lanes 5–8*) were analyzed alongside chromatin-bound proteins (Fig. 5*A*, *lanes 1–4*) using Western blotting. After a 30-min incubation in egg cytosol, ~30 fg of ORC2 bound to each sperm (Fig. 5*A*, compare *lanes 1* and *5*, *lower panel*). Assuming 2.9×10^9 bp per sperm, this is equivalent to one ORC complex per ~10.6 kb of DNA. In contrast, the average density of MCM3 was one per 275 bp (Fig. 5*A*, compare *lanes 1* and *8*, *upper panel*). The ratio of chromatin-bound MCM to ORC complexes was therefore ~40:1, similar to previous reports (23, 24). When sperm chromatin was incubated with *Xenopus* egg cytosol followed by NPE containing aphidicolin, Cdc45 loaded at an average density of 1 per 7 kb (Fig. 5*A*, compare *lane 2* and *9*), and the ratio of MCM3 to Cdc45 was ~24:1. In controls containing geminin or $p27^{Kip}$, Cdc45 binding was not observed, demonstrating that Cdc45 was bound specifically (Fig. 5*A*, *lanes 3* and *4*). The presence of aphidicolin served to prevent replication-mediated displacement of Cdc45 and did not significantly influence the amount of Cdc45 loaded (Ref. 41 and data not shown). Thus, consistent with the data in Fig. 4*C*, only a fraction of bound MCM complexes normally recruits Cdc45 during DNA replication.

Chromatin Binding by Cdc45, but Not MCM2–7, Is Limiting for DNA Replication—Because the spacing of Cdc45 on chromatin (average of 1 per 7 kb) is similar to the experimentally determined replicon size in *Xenopus* egg extracts, we asked whether chromatin binding by Cdc45 is rate-limiting for DNA replication. Sperm chromatin was mixed with Cdc45-depleted egg cytosol to assemble pre-RCs and supplemented with mock-depleted and Cdc45-depleted NPE that were mixed in different ratios. After addition of NPE, we measured the rate of DNA replication, as well as the amount of Cdc45 binding. As shown in Fig. 5*B*, the efficiency of DNA replication was proportional to the amount of Cdc45 in the extract. Importantly, there was also a strict correlation between the efficiency of DNA replication and the amount of Cdc45 bound to chromatin (Fig. 5*B*, *lower panel*). Therefore, chromatin binding by Cdc45 is limiting for DNA replication.

Experiments in nuclear assembly egg extracts previously indicated that the majority of chromatin-bound MCM complexes are not required for efficient DNA replication (23). To test if this is also true in our system, MCM2–7-depleted (11) and mock-depleted extracts were mixed in different ratios and incubated with sperm chromatin. As shown in Fig. 5*C*, when the amount of MCM complex dropped below 50% in the extract, the amount of MCM bound to chromatin began to decline, and in extracts containing only 6% the normal level of MCM complex, chromatin-bound MCM complexes were barely detectable (Fig. 5*C*, *lanes 2–5*, *top* and *middle panels*). When these extracts were supplemented with NPE, the efficiency of DNA replication was independent of the amount of chromatin-bound MCM complex (Fig. 5*C*, *graph*). MCM7-depleted extracts that were not supplemented with mock-depleted extract exhibited no binding by the MCM complex, and they did not support DNA replication (data not shown). Like DNA replication, the amount of Cdc45 that loaded onto chromatin was essentially independent of the amount of chromatin-bound MCM complex. In summary, chromatin binding of Cdc45 is rate-limiting for DNA replication, whereas MCM2–7 is not.

Cdc7-dependent Phosphorylation of Lateral MCM2–7 Complexes—Although only a small subset of chromatin-bound MCM2–7 complexes underwent activation by Cdc45, it was possible that all the MCM2–7 complexes were activated at a step that precedes Cdc45 binding. We therefore examined phos-

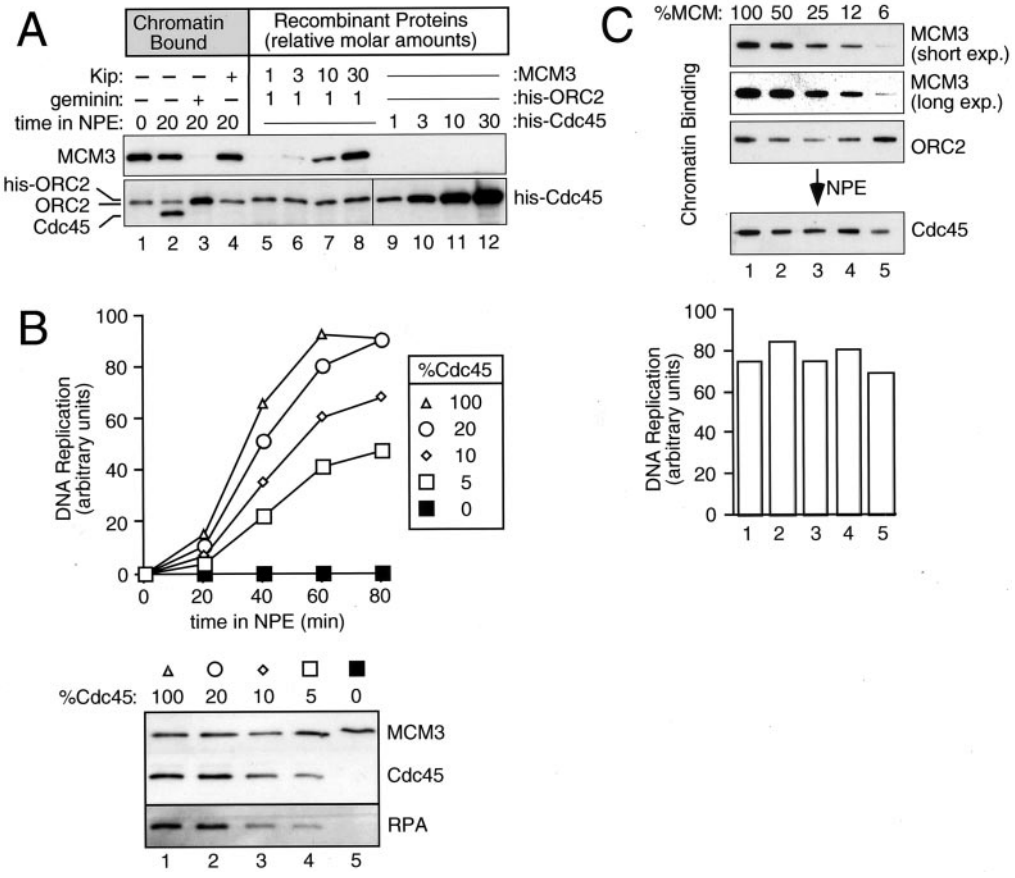

FIG. 5. **Unlike binding of MCM2–7, chromatin binding of Cdc45 is rate-limiting for DNA replication.** *A*, sperm chromatin (10,000/μl) was incubated with egg cytosol containing (*lane 3*) or lacking (*lanes 1*, *2*, and *4*) geminin and then supplemented with 2 volumes of NPE containing 50 μg/ml aphidicolin (*lanes 2–4*) and $p27^{Kip}$ (*lane 4 only*). Immediately before (*lane 1*), or 20 min after the addition of NPE (*lanes 2–4*), sperm chromatin was isolated, and the equivalent of 10,000 sperm was analyzed on a Western blot using MCM3 antibodies (*top panel*) or a mixture of ORC2 and Cdc45 antibodies (*bottom panel*). The Western blot also contained purified proteins (*lanes 5–12*). *Lane 5*, 0.4 ng of ORC2, 0.6 ng of MCM3; *lane 6*, 0.4 ng of ORC2, 1.8 ng of MCM3: *lane 7*, 0.4 ng of ORC2, 6 ng of MCM3; *lane 8*, 0.4 ng of ORC2, 18 ng of MCM3; *lane 9*, 0.4 ng of Cdc45; *lane 10*, 1.2 ng of Cdc45; *lane 11*, 4 ng of Cdc45; *lane 12*, 12 ng of Cdc45. *B*, sperm chromatin was incubated with 6 μl of Cdc45-depleted egg cytosol and then supplemented with 12 μl of total of mock-depleted and Cdc45-depleted NPE mixed in the following ratios: 1:0 (*triangles*), 1:4 (*circles*), 1:9 (*diamonds*), 1:19 (*squares*), 0:1 (*filled squares*). 9 μl of each sample was mixed with [α-^{32}P]dATP and replication measured 20, 40, 60, and 80 min after NPE addition (*graph*). The other 9 μl of each sample was mixed with aphidicolin, and chromatin binding of MCM, Cdc45, and RPA was measured 25 min after NPE addition. *C*, mock-depleted and MCM7-depleted egg cytosol were mixed in 1:0 (*lane 1*), 1:1 (*lane 2*), 1:3 (*lane 3*), 1:7 (*lane 5*), and 1:15 (*lane 6*) ratios and incubated with sperm chromatin (10,000/μl). Immediately before (*top 3 panels*) or 15 min after addition of NPE (*bottom panel*), sperm chromatin was isolated, and bound proteins were analyzed by Western blotting with antibodies against MCM3, ORC2, or Cdc45. An aliquot of each reaction was supplemented with [α-^{32}P]dATP and replication measured 60 min after addition of NPE (*bar graph*).

phorylation of the MCM2–7 complex by Cdc7, a step that is upstream of the Cdk2-dependent loading of Cdc45 (5, 6). It was reported previously (42, 43) that chromatin-bound MCM4 is phosphorylated by a Cdk2-independent protein kinase that requires nuclear assembly for activity. Analogously, in soluble *Xenopus* egg extracts, chromatin-bound MCM4 becomes phosphorylated only after addition of NPE, and this phosphorylation requires Cdc7,[2] affording us an opportunity to examine whether lateral MCM complexes can be phosphorylated in a Cdc7-dependent fashion. Fig. 6 shows that on sperm chromatin that contains a ~20:1 ratio of MCM3:ORC2 (compare *lane 6* with *lanes 7–9*), MCM4 is almost completely converted to the phosphorylated form within 4 min after the addition of NPE (Fig. 6, compare *lanes 1* and *2*). Consistent with the action of Cdc7 on lateral MCM2–7 complexes, the binding of Cdc7 to chromatin (5, 6) was geminin-sensitive and proportional to the length of DNA fragments immobilized on magnetic beads (Fig. 3*C*, *panel IV*, *lanes 1* and *2*). Together, these results suggest that all chromatin-bound MCM complexes are bound and modified by Cdc7.

[2] P. Peterson, D. Chou, T. A. Prokhorova, J. C. Walter, and G. Walter, unpublished results.

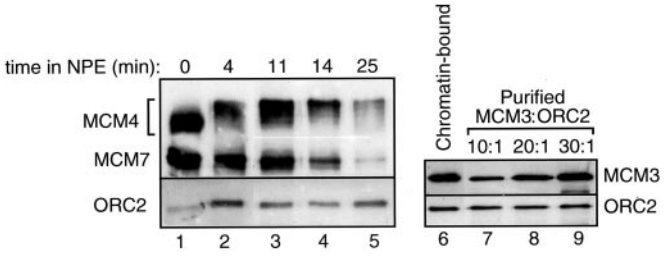

FIG. 6. **All chromatin-bound MCM complexes are phosphorylated in a Cdc7-dependent fashion.** *Xenopus* sperm chromatin was incubated with egg cytosol (10,000/μl) for 30 min and then supplemented with 2 volumes of NPE. Aliquots containing 40,000 sperm were withdrawn immediately before (*lane 1*) or at the indicated times after NPE addition (*lanes 2–7*), isolated, and the bound proteins analyzed by Western blotting using antibodies against MCM4 and MCM7 (*top panel*) or ORC2 (*bottom panel*). *Lane 6* contained the same sample as *lane 1*, and *lane 9* contained a 30:1 mixture of MCM3 (22.5 ng) to ORC2 (0.5 ng). *Top panel*, probed with MCM3 serum; *bottom panel*, probed with ORC2 serum.

Cdc45 Binding to Chromatin Is Stimulated by Actinomycin D—Our data suggest that of the many chromatin-bound MCM2–7 complexes, most are normally acted on by Cdc7, but

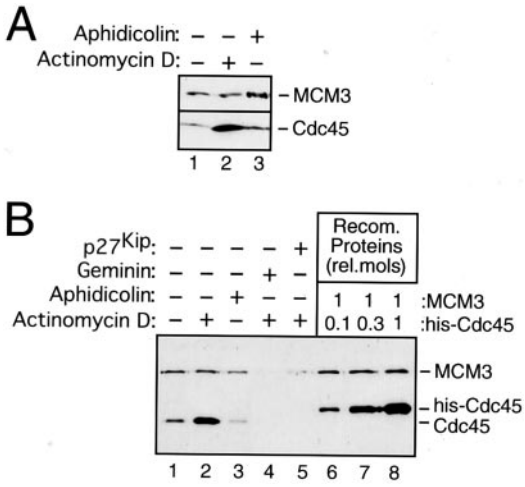

FIG. 7. **Actinomycin D stimulates Cdc45 binding.** *A,* sperm chromatin was incubated with egg cytosol and then supplemented with NPE containing buffer (*lane 1*), 10 μg/ml actinomycin D (*lane 2*), or aphidicolin (*lane 3*). After 20 min, chromatin was isolated and probed with MCM3 and Cdc45 antibodies. *B*, sperm chromatin was incubated with egg cytosol containing (*lane 4*) or lacking (*lanes 1–3* and *5*) geminin. Subsequently, NPE containing 10 μg/ml actinomycin D (*lanes 2*, *4*, and *5*), aphidicolin (*lane 3*), and $p27^{Kip}$ (*lane 5*) was added. After 20 min, chromatin was isolated and probed by Western blotting with antibodies against MCM3 and Cdc45 alongside 10 ng of purified MCM3 and 0.66, 2.2, and 6.6 ng of purified his-Cdc45 (*lanes 6–8*).

only a few recruit Cdc45. To address whether all chromatin-bound MCM2–7 complexes are competent to be activated by Cdc45, we sought to identify conditions in which most bound MCM2–7 complexes would recruit Cdc45. We speculated that a step downstream of Cdc45 binding may inactivate neighboring MCM complexes and thereby inhibit further Cdc45 binding. Therefore, we used several approaches to inhibit DNA replication after the Cdc45 loading step, and we asked whether Cdc45 binding was stimulated. Inhibition of DNA polymerase α by aphidicolin allowed Cdc45 binding but did not stimulate its binding (Fig. 7*A*, compare *lanes 1* and *3*) (10, 41). In contrast, actinomycin D, an inhibitor of the primase associated with DNA polymerase α (44), led to a large stimulation of Cdc45 binding (Fig. 7*A*, compare *lanes 1* and *2*). Importantly, in the presence of actinomycin D, Cdc45 binding was still completely dependent on the presence of chromatin-bound MCM complex (Fig. 7*B*, compare *lanes 2* and *4*) and Cdk2 activity (Fig. 7*B*, compare *lanes 2* and *5*). In the presence of actinomycin D, the Cdc45:MCM3 ratio was between 0.5 and 1 (Fig. 7*B*, compare *lane 2* with *lane 8*), suggesting that most or all chromatin-bound MCM complexes interacted with Cdc45. Because actinomycin D is a DNA intercalating agent, it is uncertain whether the observed stimulation of Cdc45 binding is due to inhibition of RNA primase or due to other effects on chromatin structure. Regardless of the mechanism, the ability of actinomycin D to induce binding of a large number of Cdc45 molecules, and the fact that this binding was Cdk2, MCM2–7, and presumably Cdc7-dependent, suggests that the majority of chromatin-bound MCM2–7 complexes are competent to support replication initiation.

DISCUSSION

The ratio of chromatin-bound MCM2–7 complexes to active origins in *Xenopus* egg extracts is ~40:1 (23, 24) (Fig. 5*A*). Using binding to immobilized linear DNA fragments of variable length, we provide evidence that this high MCM:ORC ratio comes about because MCM complexes bind to chromatin in a distributed pattern surrounding the ORC. We further show that most chromatin-bound MCM complexes are not required for efficient DNA replication (see also Ref. 23). Consistent with this, only a small subset of bound MCM2–7 complexes is normally activated by Cdc45, and unlike MCM binding, Cdc45 binding is limiting for efficient DNA replication. However, the data also suggest that the majority of lateral MCM complexes are competent to initiate DNA replication. We propose that in *Xenopus* egg extracts, origins of replication consist of multiple, distributed, initiation-competent MCM complexes (Fig. 8).

Mechanism of MCM Binding to Chromatin—ORC and the MCM complex bound in a 1:1 molar ratio to an 82-bp fragment of DNA. When MCM binding was inhibited with geminin, ORC still required ~80 bp of DNA for loading (Fig. 2*C*) (data not shown). This result is similar to a recent report (45) that the ORC complex from the fly *Sciara coprophila* occupies at least 80 bp of DNA. Because ORC binding is required for the subsequent loading of the MCM complex, it is conceivable that the MCM complex itself occupies less than 80 bp of DNA. However, ORC-distal MCM complexes also bound roughly once per 80 bp (Fig. 3*D*), suggesting that this is the length of DNA normally occupied by the MCM complex in G_1.

When DNA fragment length was increased beyond 82 bp, the amount of bound MCM complex increased dramatically, whereas the binding of ORC did not change, indicating that MCM complexes become widely distributed on chromatin surrounding ORC. We are currently not able to determine whether MCM complexes bind to distal sites by polymerizing along DNA away from a "nucleation site" containing ORC or by being delivered directly to distant sites. The latter model would predict that delivery of each MCM complex to chromatin requires Cdt1 and Cdc6, and it seems to be consistent with the failure to observe co-occupancy of ORC and MCM on 500-bp DNA fragments by *in vivo* cross-linking in mammalian cells (22). How far away from the nucleation site are MCM complexes bound? MCM complex binding was roughly proportional to DNA fragment length up to ~3 kb, and no further increase was seen between 3 and 6 kb (Fig. 3*A*). However, when we compared a 6-kb DNA fragment to sperm chromatin, the ratio of MCM to ORC was greater on sperm (Fig. 3*B*), suggesting that in the context of native chromatin structure, binding may occur at even greater distances, and possibly throughout the entire length of the chromosome. We have begun to investigate what role chromatin structure plays in the chromatin binding of the MCM complex. We found that a high MCM:ORC ratio occurs on sperm DNA that was pre-assembled into nucleosomes, arguing that nucleosomes are not inhibitory for distributed MCM complex binding.[3]

Are Lateral MCM Complexes Initiation-competent?—In the absence of ORC, Cdc6, or Cdt1, there is no binding of any MCM complexes to chromatin (39, 46). Moreover, ORC-distal MCM complexes are bound at least as tightly to DNA as ORC proximal complexes (Fig. 4*A*). Together, these data strongly argue that the chromatin binding of ORC-distal complexes has a purpose. Importantly, it was shown previously (38, 40) that once the MCM complex is loaded, ORC could be removed without any decrease in subsequent initiation efficiency. Therefore, it was conceivable that ORC-distal MCM complexes could be competent for replication initiation. This possibility is further supported by the following observations. First, lateral MCM complexes were bound by Cdc7 (Fig. 3*C*), and chromatin-associated MCM4 was quantitatively phosphorylated in a Cdc7-dependent reaction (Fig. 6). Second, in the presence of actinomycin D, the Cdc45:MCM ratio on chromatin approached 1:1,

[3] M. C. Edwards, A. V. Tutter, C. Cvetic, C. H. Gilbert, T. A. Prokhorova, and J. C. Walter, unpublished results.

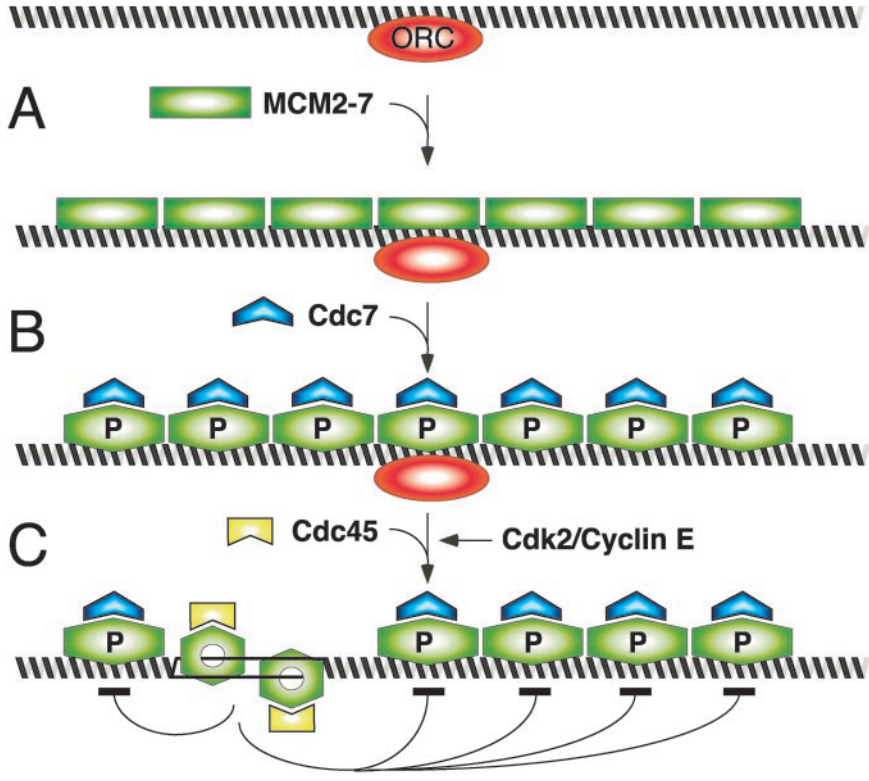

FIG. 8. **Model for the initiation of DNA replication.** *A*, ORC, Cdc6, and Cdt1 stimulate MCM2–7 binding to sites widely distributed around ORC. *B*, at the G_1/S transition, Cdc7 binds to and phosphorylates many MCM complexes. *C*, Cdk2/cyclin E stimulates the association of Cdc45 with a subset of these MCM complexes. Activation of the first MCM complexes by Cdc45 may lead to inactivation of neighboring MCM complexes, thereby restricting initiation to defined intervals.

arguing that each lateral MCM complex was competent to recruit Cdc45. Our results are consistent with two-dimensional gel analyses suggesting that MCM complexes located at a distance from a positioned ORC undergo replication initiation.[4] If lateral MCM complexes are functional, why are more Cdc45 molecules not normally recruited? We speculate that a mechanism exists to ensure that once a pair of MCM complexes is activated by Cdc45, neighboring MCM complexes up to a certain distance are rendered inactive for further initiation (Fig. 8*C*).

Significance for the "Random Completion" Problem—It is presently enigmatic how faithful genome duplication is achieved during the early cell divisions in *Xenopus* embryos (2). S phase is ~15 min long, and the replication fork moves at ~0.5 kb/min. Because there is no S phase checkpoint, any replicon greater than ~15 kb is expected to cause a mitotic catastrophe. In the absence of genetically defined origins, it is unclear how replicon size is kept below 15 kb (2). Remarkably, when several hundred eggs are fertilized *in vitro*, the vast majority of them develop normally,[5] demonstrating that an efficient solution exists. Some experiments indicate that initiation sites, although selected in a sequence-independent fashion, are regularly spaced 5–15 kb apart (47, 48). However, some 300,000 initiation events take place during each S phase, and it is difficult to imagine that every initiation event is properly spaced and occurs with 100% efficiency. Another proposal is that initiation events are randomly spaced, but that the frequency of initiations increases late in S phase (30, 49). This model is attractive, yet it appears to conflict with the maxim that new pre-replication complexes cannot be established in S phase. Our data provide a potential solution to this problem. Thus, if MCM complexes become distributed at high frequency along the length of the chromosome in G_1, any locus could undergo very frequent initiations late in S phase because no new pre-RCs must be assembled. We believe that both of the above models could be correct. Thus, most initiation events may be regularly spaced due to inactivation of neighboring MCM complexes up to a specified distance when Cdc45 loads. However, at loci where a large stretch of DNA remains unreplicated late in S phase, initiation frequency would increase to ensure completion of replication before mitosis.

Widely Distributed MCM Binding in Mammalian Cells?—There is considerable evidence that MCM complexes are widely distributed on mammalian chromosomes. The number of MCM complexes in human nuclei is ~10^6, the majority of which are chromatin-bound (25, 27, 29). Therefore, chromatin-bound MCM complexes are in excess of the ~25,000 initiation events that are estimated to occur in somatic cells (50). Consistent with a wide distribution of these MCM complexes, MCM and ORC do not co-localize on DNA fragments as large as 500–1000 bp in mammalian cells (22). In Chinese hamster ovary cells, the amount of MCM2 that associates with chromatin increases throughout G_1 phase, reaching a peak at the G_1/S transition (18). However, the efficiency of DNA replication of early and late G_1 hamster nuclei is the same when these are transferred to ORC-depleted *Xenopus* egg extracts (51). These data suggest that an excess of MCM complexes loads onto chromatin. In immunofluorescence experiments, MCM subunits are never found to co-localize with sites of DNA synthesis (18–21, 27). This is expected if only a small subset of MCM complexes that are widely distributed on chromatin is normally activated during S phase. Interestingly, at loci such as dihydrofolate reductase, initiation events are distributed over several tens of kilobase pairs (1). We speculate that this could be due to stochastic initiation from widely distributed MCM complexes.

Are MCM complexes widely distributed on the chromosomes of *S. cerevisiae*? The number of MCM complexes bound to chromatin in this organism exceeds the number of active origins by 15-fold or more, and binding of all these MCM complexes requires Cdc6 (26, 28). The binding patterns of MCM and ORC *in vivo* have been studied by chromatin immunoprecipitation (52, 53). The highest resolution experiments showed that the MCM complex binds to autonomously replicating se-

[4] J. Newport, personal communication.
[5] M. Kirschner, personal communication.

quence 305 but not to a DNA fragment located at a distance of 2 kb (53). This experiment would set an upper limit for the extent of spreading at this origin, although it cannot be ruled out that laterally bound MCM complexes do not cross-link efficiently to DNA. Significantly, replication initiation point mapping indicates that there is a single preferred start site of replication at autonomously replicating sequence 1 which overlaps the ORC footprint (54). Therefore, even if MCM spreading occurs in yeast, there would appear to be preferential activation of ORC-proximal MCM complexes. This might reflect the fact that in yeast, Cdc7 recruitment to origins of DNA replication requires the ORC complex (55). In contrast, in higher eukaryotes, Cdc7 recruitment to origins is MCM complex-dependent, but ORC-independent (5), and this may explain why ORC-distal MCM complexes appear to be initiation-competent.

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DNA: REPLICATION REPAIR AND RECOMBINATION:

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