Stepwise Regulated Chromatin Assembly of MCM2–7 Proteins*

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Acquisition of the competence to replicate requires the assembly of the MCM2–7 (minichromosome maintenance) protein complex onto pre-replicative chromatin, a step of the licensing reaction. This step is thought to occur through binding of a heterohexameric MCM complex containing the six related MCM subunits. Here we show that assembly of the MCM complex onto pre-replicative chromatin occurs through sequential stabilization of specific MCM subunits. Inhibition of licensing with 6-dimethylaminopurine results in chromatin containing specifically bound MCM4 and MCM6. A similar result was obtained by interference of the assembly reaction with an MCM3 antibody. The presence of chromatin-bound MCM intermediates was confirmed by reconstitution experiments in vitro with purified proteins and by the observation of an ordered association of MCM subunits with chromatin. These results indicate that the assembly of the MCM complex onto pre-replicative chromatin is regulated at the level of distinct subunits, suggesting an additional regulatory step in the formation of pre-replication complexes.

Studies on the mechanisms that regulate the firing of DNA replication origins have revealed that both in vitro and in vivo, chromosomes acquire the competence to replicate (the licensing reaction) on exit from metaphase (1–4). A number of proteins implicated in the licensing reaction have been identified. These include ORC1 (5–7), the protein (5, 8) CDC6, and the MCM2–7 protein complex (9–12). The licensing reaction can be divided into three separate steps. First, ORC is assembled onto chromatin, followed by CDC6 and then the MCM complex. The loading of the MCM complex requires the previous binding of ORC and CDC6 (5–7). Yeast homologues of these proteins have been shown to assemble at DNA replication origins in a similar sequential order (13–15).

The licensing reaction can be blocked with 6-DMA, a general inhibitor of serine/threonine protein kinases. When 6-DMA is added to metaphase-arrested Xenopus egg extracts, chromosomal DNA replication is blocked, very likely at the initiation step (1, 16–18). The replication defect caused by 6-DMA is efficiently rescued by a crude preparation of MCM proteins (9), indicating that 6-DMA may affect the function of MCM proteins. A first analysis of chromatin assembled in 6-DMA-treated extracts has revealed that this chromatin does not contain MCM3, whereas subunits of ORC (6, 19) and the CDC6 protein (5) are efficiently bound. Hence, one effect of 6-DMA is to inhibit the assembly of the MCM complex onto chromatin through an ORC- and CDC6-independent mechanism.

The mechanism by which the MCM complex associates with chromatin is not known. Soluble MCM proteins form complexes containing the six related MCM subunits in Xenopus (10–12, 20–22) and in a variety of other eukaryotes (for review, see Ref. 23). A soluble heterohexameric MCM complex appears to be made by association of two distinct subcomplexes: one containing MCM3 and MCM5 proteins that are in tight association with each other and another containing MCM4, -6, and -7 with which MCM2 is weakly associated (12, 22, 24). Analysis of MCM proteins in different systems has suggested that discrete MCM subcomplexes may exist in equilibrium with each other (25–27). Although their biological significance is still unclear, some specific roles for MCM subcomplexes are emerging. A subcomplex of MCM2, -4, -6, and -7 proteins may bind histone H3, probably tethered by MCM2, whereas a subcomplex of MCM3 and MCM5 has no affinity for this substrate (28). A DNA helicase activity has been reported to be associated with a subcomplex made of MCM4, -6, and -7 proteins in HeLa cells, and this activity appears to be inhibited by MCM2 (27). MCM4 and MCM6 may differentially contribute to the helicase activity of the MCM4-MCM6-MCM7 subcomplex (29). No helicase activity has been detected for the whole purified MCM complex (30), suggesting that distinct subunits of the MCM complex may have specific roles in DNA replication.

Here we show that discrete MCM subunits of the MCM complex are already bound to chromatin before its complete licensing for DNA replication. Chromatin obtained by interference with the licensing reaction was found to contain bound distinct subunits of the MCM complex. The presence of an intermediate step in the assembly of the MCM complex onto chromatin was confirmed by reconstitution experiments in vitro and by kinetic analysis of formation of the pre-replication complex. These observations reveal that specific MCM subcomplexes are components of pre-replicative chromatin and that the assembly of the MCM proteins is an ordered process that leads to formation of the pre-replication complex.

**EXPERIMENTAL PROCEDURES**

*Xenopus Egg Extracts—Interphase low speed supernatants (LSSs) were prepared as described (31). Interphase high speed supernatants were prepared as described (32). 6-DMA-treated extracts were prepared as described (1). Extracts were made 3% (w/v) with glycerol (1% for 6-DMA-treated extracts), frozen in liquid nitrogen in 50-μl aliquots, and stored at −80 °C. Before use, aliquots were thawed, pooled, and supplemented with an ATP regeneration system (10 mM creatine phosphate, 10 μM/ml creatine kinase, 1 mM ATP, and 1 mM MgCl2) and cycloheximide (250 μg/ml; Sigma).

Production of Antibodies and Immunoblotting—The B24 monoclonal antibody has been previously described (22, 33). The B24 antibody...*
obtained from mice ascites was purified by ammonium sulfate precipitation as described (34). Antibodies specific for Xenopus MCM proteins were a generous gift of Dr. H. Takisawa (University of Kyoto, Kyoto, Japan). The Xenopus ORC1 antibody was a gift from J. J. Blow (University of Dundee, Dundee, United Kingdom). The MCM peptide antibody was raised against a peptide bearing the Xenopus MCM signature (a motif), which is highly conserved in the MCM2–7 proteins (35). The peptide COOH-VCCIDEFDKMDMDRT-NH2 was synthesized, coupled to keyhole limpet hemocyanin as described (34), and injected into two different rabbits (500 μg/injection/rabbit). Western blot signals were detected with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation and Immunodepletion of MCM3 from Xenopus Egg Extracts—The B24 antibody was added to Xenopus egg extracts (high speed supernatant) diluted 5-fold with phosphate-buffered saline containing protease inhibitors (5 μg/ml each leupeptin, aprotinin and pepstatin; Sigma). Immunocomplexes were allowed to form by incubation on a rotating wheel at 4 °C for 30 min, protein G-Sepharose beads (Sigma) were added, and incubation was continued for 30 min under the same conditions. Immunocomplexes were recovered by low speed centrifugation and washed five times with ice-cold phosphate-buffered saline containing protease inhibitors, and bound proteins were eluted with Laemmli buffer. For immunodepletion, either the B24 antibody or control mouse IgGs were coupled to protein G-Sepharose beads (2:1, v/v) for 1 h at room temperature on a rotating wheel. Coupled IgGs were extensively washed with phosphate-buffered saline and finally with XB buffer (100 mM KCl, 0.1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES-KOH (pH 7.7), and 50 mM sucrose with protease inhibitors). One hundred microliters of Xenopus LSS were supplemented with cycloheximide and double-depleted with a 50% volume of protein G beads (Sigma) coupled to either the B24 antibody or control mouse IgGs by incubation for 40 min at 4 °C. Supernatants were recovered by low speed centrifugation at 4 °C and supplemented with an energy regeneration system, demembranated sperm nuclei as required, and [γ32P]dCTP (Amersham Pharmacia Biotech).

Immunopurification of MCM2–7 Proteins—The B24 antibody or control IgGs were covalently coupled to protein G beads as described (36). The beads were incubated with Xenopus egg extracts (LSS) for 40 min at 4 °C on a rotating wheel and separated by low speed centrifugation at 4 °C. The beads were thoroughly washed with XB buffer on ice in the presence of protease inhibitors. Proteins specifically bound to the beads were eluted first with 2 volumes of XB buffer + 1.5 mM NaCl for 10 min on ice, washed with XB buffer, and then re-eluted with 2 volumes of glycine (pH 2.5). Proteins eluted with glycine were immediately neutralized by addition of 0.1 volume of 1 M Tris (pH 8.8). Eluted proteins were simultaneously dialyzed against XB buffer and concentrated at 0.5 mg/ml by centrifugation in a Microcon-10 (Amicon, Inc.) at 4 °C. Fractions were stored as aliquots at –20 °C in 10% glycerol.

Neutralization of MCM3-Chromatin Binding—About 3 μg (2 μl) of either the B24 antibody or control mouse IgGs (eluted from protein G-Sepharose beads) were added to 30 μl of a Xenopus LSS and incubated on a rotating wheel at 4 °C for 30 min. Following incubation, extracts were supplemented with an energy regeneration system and demembranated sperm nuclei (4 μg/μl), and incubation was continued at 23 °C for 15 min. For heat inactivation, the B24 antibody was boiled for 5 min in the presence of 1 μM dithiothreitol, cooled immediately on ice, and then added to the reaction mixture. Chromatin was purified as described below.

Chromatin Purification Methods—Demembranated sperm nuclei were prepared as described (31). Chromatin reconstituted in Xenopus egg extracts was purified by the modification of a previously described protocol (37). Samples were diluted 4-fold in ice-cold chromatin purification buffer (50 mM KCl, 20 mM HEPES-KOH (pH 7.7), 2% sucrose, 5 mM MgCl2, 0.1% Nonidet P-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin). Chromatin was purified by centrifugation at 6000 × g for 5 min at 4 °C through a 1.5-ml 0.7 M sucrose cushion made in chromatin purification buffer without Nonidet P-40 in a microcentrifuge. Pellets were washed once with chromatin purification buffer, and proteins were eluted with 2× Laemmli buffer. MCM-depleted or 6-DMAP-treated chromatin was obtained by incubation of demembranated sperm nuclei (30–50 ng/μl) in the corresponding extracts for 15 min at 23 °C. Chromatin was purified as described above, except that Nonidet P-40 was omitted, and pellets were resuspended in XB buffer. Samples were frozen as 5–μl aliquots in liquid nitrogen and stored at –80 °C as described (6).

Reconstitution of Unlicensed Chromatin with Purified MCM Proteins in Vitro—MCM-depleted or 6-DMAP-treated chromatin was reconstituted in vitro with purified MCM subcomplexes as follows. About 10 ng of MCM-depleted chromatin or 20 ng of 6-DMAP-treated chromatin were incubated with fractions eluted from the B24 immune complex (1 μl) for 15 min at 23 °C. After incubation, 1 μl of the reaction was mixed with 9 μl of either MCM- or mock-depleted Xenopus interphasic extract (LSS), and DNA replication was monitored over 90 min at 23 °C. In vitro reconstituted chromatin was purified as described in the specific chromatin purification section.

Immunofluorescence Microscopy—Chromatin formed in Xenopus egg extracts was fixed and observed by immunofluorescence microscopy as described (37) using an inverted Zeiss Axioscope.

RESULTS

Assembly of MCM4 onto Chromatin Can Be Dissociated from That of MCM3—MCM proteins associate with chromatin very rapidly on exit from metaphase (4). Using Xenopus DNA replication in vitro systems, we had previously observed that the MCM4 protein associates with chromatin more rapidly than MCM3 (22). To test whether MCM4 would bind to chromatin independently of MCM3, we interfered with the association of MCM3 with chromatin using a specific monoclonal antibody (see “Experimental Procedures”). This antibody immunoprecipitated MCM3 (Fig. 1A) and also efficiently removed the five related MCM proteins from Xenopus S-phase egg extracts (Fig. 1B), consistent with these proteins forming a stable complex in solution (10, 12, 22). When the MCM3-specific antibody was added to an interphasic Xenopus egg extract, the association of MCM3 with chromatin was severely inhibited (Fig. 1C, lane 2), whereas addition of control IgGs did not have any effect (lane 1). The presence of the MCM3-specific antibody also caused inhibition of DNA replication, as expected when chromatin is not fully licensed (Ref. 13 and data not shown). The binding of MCM3 to chromatin was not affected when the antibody was
heat-inactivated (lane 3) or once licensing had already occurred, i.e. 15 min after addition of sperm chromatin (lane 4). In contrast to what was observed with MCM3, MCM4 could still bind chromatin under these conditions (Fig. 1C), as could ORC1 (Fig. 1C), which is not complexed with MCM proteins in solution (6, 7). We conclude that MCM4 binds to chromatin when the binding of the MCM3 protein is specifically blocked, demonstrating that MCM4 can associate with chromatin independently of MCM3.

**MCM4 and MCM6 Proteins Are Specifically Associated with Unlicensed 6-DMAP-treated Chromatin**—In Xenopus, the association of the MCM3 protein with chromatin can be blocked with 6-DMAP, an inhibitor of serine/threonine protein kinases, resulting in inhibition of the initiation of DNA replication (1, 5, 6). 6-DMAP inhibits the activity of the licensing factor, which normally limits DNA replication to only one round in each S-phase (1). We wished to determine whether the binding of MCM4 to chromatin could be affected by 6-DMAP as supplementary evidence that MCM4-chromatin binding can be uncoupled from that of MCM3. We have biochemically characterized chromatin obtained in Xenopus egg extracts treated with 6-DMAP. Addition of 6-DMAP to metaphase-arrested extracts inhibits by >90% DNA replication following release into interphase (Ref. 1 and data not shown). The presence of 6-DMAP completely abolished the association of MCM3 with chromatin, as expected (Fig. 2A); however, the MCM4 protein was found to be specifically bound to 6-DMAP-treated chromatin. The specific binding of MCM4 to 6-DMAP-treated chromatin was also confirmed by immunofluorescence microscopy with specific antibodies (Fig. 2C). Previous characterization of chromatin formed in 6-DMAP-treated extracts has shown that the ORC1, ORC2, and CDC6 proteins are bound (5, 6, 19), but it is not known whether 6-DMAP interferes with the binding of all MCM subunits. Analysis of MCM proteins bound to 6-DMAP-treated chromatin showed that in addition to MCM3, the binding of MCM2, -5, and -7 proteins is also largely repressed. Surprisingly, we observed that MCM6 could be recovered on this chromatin at almost physiological levels (70% of the control) (Fig. 2, A and B).

We conclude that 6-DMAP does not inhibit the association of two MCM subunits (MCM4 and MCM6) with chromatin and that a whole MCM complex is not required for the retention of these subunits on chromatin. One implication of these observations is that the MCM4-MCM6 subcomplex may be a stable intermediate of the licensing reaction.

**Reconstitution of Unlicensed Chromatin with Purified MCM Proteins in Vitro**—To determine whether formation of a heterohexameric MCM2–7 complex is required for the association of distinct MCM subunits with chromatin, we performed reconstitution experiments in vitro with MCM subcomplexes isolated from interphase, replication-competent Xenopus egg extracts. These were obtained by stepwise elution of the MCM2–7 complex immunopurified with the MCM3-specific monoclonal antibody. The first fraction, obtained by high salt wash, contained the MCM3 subcomplex (Fig. 3A, Eluates, 1.5M NaCl), whereas the second fraction, obtained by an additional wash at low pH (Glycine), contained the MCM3-MCM5 subcomplex. These purified fractions were incubated in vitro either with chromatin lacking all MCM subunits (MCM-dep chr) or with chromatin assembled in the presence of 6-DMAP (6-DMAP chr; see the experimental procedure outlined in Fig. 3B). The ability of the MCM3 protein to bind chromatin was determined by Western blotting. Incubation of the total MCM complex with either MCM-depleted or 6-DMAP-treated chromatin restored the binding of MCM3 (Fig. 3C). The MCM3-MCM5 subcomplex was not sufficient to allow binding of MCM3 to chromatin lacking all MCM subunits. In contrast, the same MCM3-MCM5 subcomplex was sufficient to restore binding of MCM3 to 6-DMAP-treated chromatin, which, as we have shown (Fig. 2), already contains bound MCM4 and MCM6 proteins. These results indicate that the isolated MCM3-MCM5 subcomplex only binds to chromatin if the MCM4 and MCM6 proteins are bound. The replication competence of in vitro reconstituted chromatin was determined by incubation in an extract lacking MCM proteins (Fig. 5D). As expected, chromatin lacking MCM proteins (MCM-dep chr) did not replicate in an extract that did not contain MCM proteins (Fig. 5D, bar 2) compared with a control incubation in a mock-depleted extract (bar 1). The total MCM complex did stimulate replication of both MCM-depleted chromatin (bar 3) and 6-DMAP-treated chromatin (bar 6), demonstrating that the purified MCM complex is functional. In contrast, the MCM3-MCM5 subcomplex did not stimulate the replication of 6-DMAP-treated chromatin (bar 7), despite the fact that the binding of MCM3 was restored (Fig. 3C). Thus, rescue of DNA replication in MCM-depleted extracts is obtained only by re-addition of all six MCM subunits, confirming previous observations (11). Collectively, these results indicate that (a) the association of the MCM3-MCM5 subcomplex with
are the results from DNA replication of in vitro extract as a control. MCM-depleted chromatin was also MCM-depleted (Mock-dep chr), and DNA replication was measured by Western blotting with the MCM peptide antibody (data not shown).

.binding of the MCM subunits not indicated in the figure was confirmed by Western blotting with specific antibodies (αMCM6, αMCM4, and αMCM3). Numbers on both the right- and left-hand sides indicate MCM proteins. The asterisk indicates a polypeptide that was recognized by the antibody and may represent a degradation product. HSS, high speed supernatant; B, shown is the experimental procedure of the reconstitution experiment. Either MCM-depleted chromatin (MCM-dep chr) or 6-DMAP-treated chromatin (6-DMAP chr) is incubated in vitro either with the MCM3/MCM5 subcomplex or the MCM complex. After 15 min at 23 °C, one aliquot of each sample is incubated with an MCM-depleted extract, whereas reconstituted chromatin is purified from another aliquot. C, shown are the results from in vitro binding of MCM3 to chromatin reconstituted with MCM complexes. Western blot of chromatin formed in MCM-depleted extracts (MCM-dep chr) or 6-DMAP-treated extracts (6-DMAP chr) reconstituted in vitro either with proteins eluted from B24 antibody beads (MCM3 and MCM5 and the MCM complex) (+) or with proteins eluted from IgG beads (−). The binding of the MCM subunits not indicated in the figure was confirmed by Western blotting with the MCM peptide antibody (data not shown). Mock-dep chr, chromatin formed in a mock-depleted extract. D, shown are the results from DNA replication of in vitro reconstituted chromatin. One aliquot of in vitro reconstituted chromatin was incubated in an MCM-depleted (MCM-dep) extract, and DNA replication was measured after 90-min incubation at 23 °C. MCM-depleted chromatin was also incubated in a mock-depleted (Mock-dep) extract as a control. Bars 1, no addition; bars 2 and 5, with proteins eluted form control IgGs; bars 3 and 6, with the MCM complex; bars 4 and 7, with the MCM3/MCM5 subcomplex.

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chromatin is dependent upon the presence of the MCM4 and MCM6 proteins; (b) a whole MCM complex is not required for the association of the MCM3 and MCM5 subunits with chromatin; and (c) the binding of MCM3 to 6-DMAP-treated chromatin is not sufficient to restore DNA replication.

Sequential Assembly of MCM Subunits onto Chromatin during the Licensing Reaction—In support of these results, we have analyzed in detail the kinetics of the association of all MCM subunits with chromatin by both indirect immunofluorescence and Western blotting with specific antibodies. The analysis was performed during the first minutes of incubation of demembranated sperm nuclei in an interphase Xenopus egg extract, i.e. before the formation of the nuclear membrane. During the first minute of the time course (Fig. 4A), MCM4 and MCM6 proteins associated with chromatin very rapidly. The MCM3 protein was detected on chromatin only at a later stage (5–10 min), and the rapid accumulation of MCM6 compared with MCM4 was confirmed by Western blot analysis of chromatin fractions (Fig. 4B). Quantification of the signals obtained by Western blotting and determination of the MCM3/MCM6 signal ratio during the kinetics of chromatin association clearly confirmed that MCM6 binds chromatin with faster kinetics than does MCM3 (Fig. 4C). These data are in agreement with the results obtained with 6-DMAP and with the in vitro reconstitution experiments showing that MCM4 and MCM6 are required to prime the assembly of the MCM complex. MCM7, which in solution forms a stable complex with MCM4 and MCM6 (22), surprisingly began to accumulate on chromatin only 5 min after incubation (Fig. 4B), suggesting that its loading or stabilization may be time-dependent. MCM5 bound to chromatin with kinetics similar to those of MCM7 (Ref. 20 and data not shown). ORC1 bound to chromatin very rapidly, as rapidly as MCM4 and MCM6 (Fig. 4B), but reached maximal

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Purification of MCM complexes from Xenopus egg extracts. **A**, MCM proteins were immunopurified from interphase Xenopus egg extracts (LSS) by incubation with the B24 antibody. Proteins eluted from beads with high salt (Eluates, 1.5M NaCl) and low pH (Glycine) were fractionated by 8% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with the MCM peptide antibody (αMCM pep). The presence of specific MCM proteins in eluates was verified by Western blotting with the MCM peptide antibody (data not shown).

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Purification of MCM complexes from Xenopus egg extracts. A, MCM proteins were immunopurified from interphase Xenopus egg extracts (LSS) by incubation with the B24 antibody. Proteins eluted from beads with high salt (Eluates, 1.5M NaCl) and low pH (Glycine) were fractionated by 8% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with the MCM peptide antibody (αMCM pep). The presence of specific MCM proteins in eluates was verified by Western blotting with the MCM peptide antibody (data not shown).

![Figure 4](http://www.jbc.org/)

**FIG. 4.** Dynamics of MCMs binding to chromatin during the licensing reaction. A, demembranated sperm nuclei (3 ng/µl) were incubated in a Xenopus interphase extract (LSS) for the indicated times. Chromatin samples were fixed, and bound proteins were visualized by in situ indirect immunofluorescence with specific antibodies. Scale bar = 10 μm. B, kinetics of MCM protein binding to unlicensed chromatin analyzed by Western blotting of chromatin fractions. A sample of chromatin formed during the licensing reaction (A) was purified, and bound proteins were analyzed by Western blotting with the indicated antibodies. C, MCM6 binds to sperm chromatin earlier than MCM3. Western blot signals of MCM3 and MCM6 shown in B were quantified and are expressed as the MCM3/MCM6 signal ratio versus time.
levels of accumulation before chromatin was fully loaded with all MCM proteins. The very rapid accumulation of ORC1 is consistent with the notion that ORC is required for the association of MCM proteins with chromatin (5–7).

We conclude that MCM2–7 proteins associate with chromatin with distinct kinetics. MCM4 and MCM6 are the first MCM proteins to accumulate significantly on chromatin at an early step in the licensing reaction, with kinetics similar to those of ORC1.

**DISCUSSION**

**MCM Subcomplexes as Intermediates of the Licensing Reaction**—In this study, we report the first comprehensive analysis of the assembly of the MCM2–7 complex onto pre-replicative chromatin. In contrast with the observation that all six MCM proteins are associated in a complex in solution and are therefore expected to bind chromatin as a whole heterohexameric complex, we have revealed the existence of discrete subunits of the MCM complex bound to chromatin during formation of the pre-replication complex. Only once formation of pre-replication complexes had been completed were all MCM proteins observed on chromatin. We have identified MCM4 and MCM6 proteins as a stable intermediate of the assembly of the MCM2–7 proteins onto pre-replicative chromatin. This intermediate was observed at an early step in the licensing reaction and also when the licensing reaction was blocked with 6-DMP. Hence, 6-DMAP may interfere with a phosphorylation event required for either the assembly or the stabilization of a subset of MCM proteins on chromatin, but the binding of MCM4 and MCM6 does not depend on this regulation. Similar results were obtained by interference with the chromatin binding of MCM3 with a specific antibody, a treatment that did not prevent MCM4 from binding to chromatin. Given that MCM3 and MCM5 proteins cannot bind chromatin devoid of MCM4 and MCM6 subunits, the assembly of MCM4 and MCM6 proteins may represent a priming step in the chromatin assembly of the MCM proteins. The inability of the MCM3/MCM5 subcomplex to bind chromatin lacking all MCM subunits also indicates that this subcomplex has low affinity for chromatin and is consistent with previous observations showing that an MCM3/MCM5 subcomplex from HeLa cells has no affinity for histones (28).

The *in vitro* reconstitution experiments confirm that isolated MCM subcomplexes can bind chromatin, suggesting that formation of a heterohexameric complex made of the six MCM subunits is not required for the binding of MCM proteins or for their retention on chromatin. They also indicate that a full MCM complex is required for DNA replication in *Xenopus* egg extracts, in agreement with previous findings showing that all six MCM proteins are required to rescue DNA replication in MCM-depleted extracts (10, 11, 20) and that a crude preparation of MCM proteins (replication licensing factor-M) can rescue the replication defect of 6-DMAP-treated extracts (9). However, purified replication licensing factor-M appears to require an additional fraction, replication licensing factor-B, to restore licensing in 6-DMAP-treated extracts (9). Our results suggest that replication licensing factor-B may be an activity needed to assemble and/or stabilize part of the MCM complex on chromatin. Purification and characterization of this activity will be of great interest to elucidate the regulation of assembly of the MCM proteins onto chromatin.

**Dynamics of MCM2–7 Protein Assembly onto Chromatin**—Analysis of the assembly of the MCM proteins onto chromatin during the licensing reaction has revealed that MCM proteins are loaded in at least two steps. The first step (1–3 min) involves the rapid binding of MCM4 and MCM6 as well as MCM2 (22). This step correlates with the accumulation of ORC1 on chromatin, raising the possibility that at this stage of the licensing reaction, ORC1 and the MCM2, -4, and -6 proteins may physically interact. A physical interaction between ORC1 and MCM4 has been reported in whole cell extracts of fission yeast (38). The assembly of this intermediate may represent a pre-licensing step that is not inhibited by 6-DMP. In the second step (>5 min), all MCM proteins accumulate at high levels on chromatin, suggesting that this step may be dependent on pre-licensing. Given that all MCM subunits are required for license for replication (Refs. 11 and 12 and this work), these observations may explain why demembranated sperm nuclei acquire the “license” to replicate only after 15 min of incubation in *Xenopus* interphase extracts (1), as this is the minimal time required to complete the third step in the licensing reaction, i.e. the assembly of all MCM2–7 subunits onto chromatin.

MCM7 accumulated on chromatin only at a later stage of the licensing reaction, suggesting that it may require either prior binding of other MCM subunits or a stabilization event that is time-dependent. This finding is consistent with the absence of MCM7 in 6-DMP-treated chromatin and strengthens the notion that 6-DMP interferes with a step in the assembly of a subset of MCM subunits. In solution, MCM7 forms a strong complex with two other MCM proteins, MCM4 and MCM6 (12, 22); and this complex, purified from HeLa cells, has been shown to have DNA helicase activity, which may be relevant to the unwinding step of initiation of DNA replication (27). The helicase activity of the MCM4-MCM6-MCM7 subcomplex is inhibited by addition of the MCM2 protein, and no helicase activity is associated with a heterohexameric complex made of the six distinct MCM subunits (30). We speculate that formation of the MCM4-MCM6-MCM7 complex on chromatin is regulated, perhaps being remodeled in an active form only after formation of the nuclear membrane, when S-phase cyclin-dependent kinases are active and initiation of DNA replication is allowed.

The evidence presented here shows that MCM2–7 proteins are loaded onto chromatin sequentially, whereas in solution, these proteins are associated in a heterohexameric complex. We envisage two models that can explain how MCM2–7 proteins are loaded onto pre-replicative chromatin. In the first model, MCM subunits are delivered to chromatin from the soluble MCM complex, perhaps with the collaboration of “chaperone-like” proteins. In the second model, MCM proteins bind to chromatin as a heterohexameric complex, but during the licensing reaction, this complex is unstable. MCM subunits with high affinity for chromatin targets (*e.g.* MCM4 and MCM6; see also Ref. 29) would be the first to be stabilized, whereas others would be more sensitive to dissociation. We favor the second model, which is supported by our *in vitro* reconstitution experiments showing that purified MCM proteins do not need to be assembled into a full heterohexameric complex in order to bind chromatin. This second model is consistent with a model recently proposed for the loading of MCM2–7 proteins onto pre-replicative chromatin in yeast (39), which parallels the loading of proliferating cell nuclear antigen, a DNA polymerase-δ processivity factor. Proliferating cell nuclear antigen in solution is a ring-shaped homotrimeric protein that binds to the DNA substrate, forming an unstable intermediate. ATP hydrolysis by the replication factor C complex causes a conformational change that promotes opening of the proliferating cell nuclear antigen ring and its reformation around DNA, a reaction that is highly regulated (40, 41). Subunits of ORC and the CDC6 protein, which are required for the loading of MCM2–7 proteins onto chromatin, share significant sequence homology with subunits of replication factor C, and genetic evidence in yeast suggests that CDC6 requires ATP hydrolysis to load MCM proteins onto chromatin (39, 42, 43). Thus, the soluble hetero-
hexameric MCM complex, which appears to form a ring in solution (30), may contact chromatin via specific MCM subunits (MCM2, -4, and -6), forming an unstable intermediate. A phosphorylation event sensitive to 6-DMAP and ATP hydrolysis, presumably mediated by ORC and CDC6, will be required to stabilize the MCM complex on chromatin, completing the licensing reaction.

The observation of the assembly of MCM proteins onto chromatin through distinct MCM subcomplexes suggests an additional level of regulation for the initiation of DNA replication. One intermediate step in the assembly of MCM proteins is sensitive to 6-DMAP, suggesting that it is regulated by the activity of protein kinase(s). These data also raise the question of whether MCM proteins reassemble in a whole heterohexameric complex onto chromatin or whether they distribute differentially at distinct sites, perhaps dynamically interacting in an ordered manner at discrete steps in the initiation reaction. The identification of the chromatin targets for MCM proteins and the analysis of their distribution on chromosomes will be of great importance in understanding the function of these universal regulators of the initiation of DNA replication in eukaryotes.

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