Sequential MCM/P1 Subcomplex Assembly Is Required to Form a Heterohexamer with Replication Licensing Activity*

(Received for publication, July 22, 1999, and in revised form, October 26, 1999)

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Replication licensing factor (RLF) is a multiprotein complex involved in ensuring that chromosomal DNA replicates only once in a single cell cycle. It comprises two components, termed RLF-M and RLF-B. Purified RLF-M consists of a mixture of complexes containing all six members of the MCM/P1 family of minichromosome maintenance proteins. The precise composition of these different complexes and their contribution to RLF-M activity has been unclear. Here we show that in Xenopus extracts, MCM/P1 proteins mainly form heterohexamers containing each of the six proteins. This heterohexamer is readily split into subcomplexes, whose interactions and subunit composition we characterize in detail. We show for the first time an ordered multistep assembly pathway by which the heterohexamer can be reformed from the subcomplexes. Importantly, this novel pathway is essential for DNA replication, since only the full heterohexamer can bind productively to chromatin and provide RLF-M activity.

Proteins belonging to the MCM/P1 family play a central role in the control of chromosomal DNA replication in eukaryotes. Current information about the gene family suggests that it consists of six closely related paralogues, termed MCM2, -3, -4, -5, -6, and -7, likely to be present in all eukaryotes (reviewed in Refs. 1 and 2), although developmentally regulated variants may also exist (3). The founding members of the family, MCM2, -3, and -5, were identified in the yeast Saccharomyces cerevisiae in a screen for minichromosome maintenance (MCM) mutants that showed origin-specific defects in the initiation of DNA replication (4). The first vertebrate member of the family to be identified was the P1 protein, a human Mcm3 orthologue that co-purified with DNA polymerase alpha (5). Since the MCM screen identified other genes such as MCM1, -10, -16, -17, -21, and -22 (6–10) that are not related to MCM2–MCM7, we have suggested the name “MCM/P1” to denote the family (1). MCM/P1 family members have now been identified in a wide range of eukaryotes, including Drosophila, Xenopus, and humans (reviewed in Ref. 2). Experiments in Drosophila, S. cerevisiae, and Schizosaccharomyces pombe suggest that each member of the MCM/P1 family is individually required for chromosomal DNA replication. Each member of the MCM/P1 family contains a putative nucleotide binding motif (11), and a complex of Mcm4, -6, and -7 has been shown to have weak helicase activity (12). However, the precise role of the MCM/P1 proteins in DNA replication remains unclear.

Replication licensing factor (RLF) was originally identified as an essential DNA replication activity that “licenses” replication origins during late mitosis or early interphase for a single initiation event, thus ensuring precise duplication of the genome (Ref. 13; reviewed in Ref. 14). Fractionation of RLF activity from Xenopus egg extracts showed that it consisted of two essential components, termed RLF-M and RLF-B (15, 16). RLF-M consists of complexes containing all six members of the Xenopus MCM/P1 family (15, 17–19). For licensing to occur, RLF-M and RLF-B must be incubated with chromatin that also contains bound ORC (the origin recognition complex) and Cdc6 (16, 20–22). This result in the MCM/P1 proteins being assembled onto chromatin. Once licensing has occurred, ORC and Cdc6 are no longer required for DNA replication (23, 24). Licensing occurs only in late mitosis and G1, but the MCM/P1 proteins are displaced from DNA as it replicates, thus ensuring that rereplication does not occur (15, 17–19, 25–29).

The six MCM/P1 proteins associate to form different high molecular weight complexes. The largest of these complexes, with an apparent molecular mass of ~600 kDa on gel filtration, could represent a heterohexamer of each of the six MCM/P1 proteins. The quantitative co-association of all six MCM/P1 family members described in Xenopus and S. pombe (18, 19, 27, 30) is consistent with this idea. However, other complexes of a comparable size are observed that do not contain all six members of the MCM/P1 family and therefore cannot be simple heterohexamers (e.g. 12, 19, 31). In mammalian cells, MCM/P1 proteins are not found as heterohexamers but instead mainly form smaller complexes (32–35). These include a heterodimer containing Mcm3 and -5 (18, 32, 33, 36, 37) and subcomplexes containing Mcm4, -6, and -7, plus or minus Mcm2 (31, 32, 34–36, 38, 39). The relationship between these various complexes and their precise composition has not been determined. More importantly, it has also been unclear which of these complexes is responsible for providing the essential RLF-M activity required for DNA replication.

In this paper, we characterize in detail the subcomplexes of MCM/P1 proteins that are formed upon chromatographic fractionation of Xenopus egg extracts. We show that MCM/P1 proteins in Xenopus egg extract exist mainly as heterohexamers, which readily dissociate into a number of distinct subcomplexes. We characterize the composition of these subcomplexes and show that they can be reassembled into heterohexamers by a specific assembly pathway. Importantly, we show that the heterohexameric complex alone provides RLF-M activity.

* This work was supported by Cancer Research Campaign Grant SP2385. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MCM, minichromosome maintenance; RLF, replication licensing factor; 6-DMAP, 6-dimethylaminopurine.

EXPERIMENTAL PROCEDURES
Preparation of Egg Extracts and Chromatin—Xenopus egg extracts were prepared as described (40, 41). 6-Dimethylaminopurine (6-DMAP)-treated extracts were prepared by supplementing metaphase-
arrested extracts with 3 mM 6-DMAP, which blocks the activation of licensing factor that normally occurs upon exit from metaphase (40, 42). Interphase extracts for gel filtration were prepared by releasing metaphase extracts into interphase by the addition of 0.3 mM CaCl2. Xenopus sperm nuclei were obtained by manual dissection of testes and were demembranated with lysolecithin as described (41). One ml of demembranated sperm nuclei was diluted to 1 ml with NIB (50 mM KCl; 50 mM Hepes KOH, pH 7.6; 5 mM MgCl2; 5 mM EGTA; 2 mM β-mercaptoethanol; 0.5 mM spermidine; 0.15 mM spermine; 1 μg/ml each leupeptin, aprotinin, and pepstatin) supplemented with 0.1% Nonidet P-40. Chromatin was then centrifuged through a 15% sucrose cushion made up in the same buffer for 5 min at 700 × g in a swing-out rotor at 4 °C. Where indicated, sperm nuclei were resuspended in a reaction buffer to which 10 μM of protein subcomplexes was added with 1 ml of 400 ng of DNA/μl was incubated with 10 μM of protein subcomplexes supplemented with 10 μM of RLF-B, and 3 μl of Q1, Q3, QH3a, or RLF-M for 30 min. The chromatin was isolated in NIB as described above and resuspended in NIB at 80 ng of DNA/μl.

RESULTS

MCM/P1 Proteins Form Heterohexameric Complexes in Xenopus Egg Extract—We first analyzed the molecular weight of the native MCM/P1 complexes in Xenopus egg extract. To obtain molecular weights that are not biased by shape, a combination of gel filtration (which overestimates the size of elongated molecules) and glycerol gradient sedimentation (which underestimates the size of elongated molecules) can be used (45). Most of the six MCM/P1 proteins migrated on gel filtration with an apparent molecular mass of ~600 kDa (Stokes radius ~77 Å, Fig. 1A), consistent with previous reports (15, 19).

Previous attempts to estimate the size of this complex by glycerol gradient centrifugation had given an anomalously low figure of ~13.5 S, either because the complex was an elongated molecule of ~400 kDa or because the complex was unstable on the glycerol gradients (19). To improve stability on the glycerol gradient, the salt concentration was lowered to 50 mM, and the glycerol concentration was reduced. This resulted in a more homogeneous peak of MCM/P1 proteins at an apparent molecular mass of ~440 kDa (~17.5 S, Fig. 1B), suggesting that the previous low value had been due to the instability of the complex.

Using the formula of Siegel and Monty (45), the new values suggest a slightly elongated complex of ~565 kDa. This figure is consistent with the 547 kDa calculated for the combined molecular masses of all six Xenopus MCM/P1 proteins (see “Experimental Procedures”).

To determine whether this complex mainly represented a heterohexamer of all six proteins, crude Xenopus extract was immunoprecipitated with antibodies against XMcm3 or XMcm7. Consistent with previous studies (18, 19, 27, 46), virtually all of the MCM/P1 proteins co-precipitated with either anti-XMcm3 or anti-XMcm7 antibodies, suggesting the presence of an XMcm(2–7) heterohexamer (Fig. 1C). When the immunoprecipitates were washed in 0.3 and 1 M salt, a separation of the MCM/P1 proteins was observed. XMcm1, XMcm2, XMcm4, and XMcm5 were displaced. Conversely, XMcm3 and -7 remained associated with XMcm3 in 1M salt, whereas most of the XMcm2, -4, -6, and -7 were removed. Similarly, XMcm4 and -6 remained associated with the XMcm7 immunoprecipitate in 1 M salt, while all of XMcm2 and most of XMcm3 and -5 were removed. This suggests that stable subcomplexes containing different MCM/P1 proteins can be formed from the hexamers.

MCM/P1 Protein Complexes Can Be Separated into Distinct Subcomplexes Chromatographically—We next investigated whether the MCM/P1 subcomplexes could be resolved by chromatography. A crude fraction of interphase Xenopus extract containing all of the MCM/P1 proteins (15, 19) was applied to a Q Sepharose column. No MCM/P1 proteins appeared in the flow-through or in the 1 M salt wash of the column. Upon elution with a shallow KCl gradient, they separated into three distinct peaks, designated Q1, Q2, and Q3 (Fig. 2A). Q1 con-
RLF-M activity were also those that provided each of the six MCM/P1 proteins.

We next investigated the composition of these subcomplexes by gel filtration and immunoprecipitation. Upon gel filtration of fraction Q1, both XMcm3 and -5 co-migrated with an apparent molecular mass of ~230 kDa (Fig. 3A). XMcm3 in this fraction sedimented on glycerol gradients with an apparent molecular mass of ~170 kDa (data not shown), suggesting a molecular mass of ~200 kDa. Immunoprecipitation of XMcm3 from the Q1 fraction co-precipitated all of the XMcm5 (Fig. 3F), suggesting that the complex mainly consists of XMcm(3 + 5) heterodimers. Consistent with this, further purification of QH1 to apparent homogeneity by hydrophobic chromatography yielded approximately equal quantities of XMcm3 and -5 as judged by Coomassie staining (Fig. 3E, lane 1). Densitometry of the bands gave the ratio XMcm3:XMcm5 as 1:0.1:2. The slight excess of XMcm5 might possibly be due to the presence of some XMcm5 homodimer. Similar results were obtained with the Q2 fraction, where XMcm3 and -7 co-migrated upon gel filtration with an apparent molecular mass of ~230 kDa (Fig. 3B). The majority of the XMcm7 was co-precipitated with anti-XMcm3 antibodies (Fig. 3F), again suggesting a heterodimer, this time of XMcm(3 + 7).

Upon gel filtration of the QH3a fraction, XMcm2, -4, -6, and -7 co-migrated as a single complex with an apparent molecular mass of ~500 kDa (Fig. 3C). Immunoprecipitation of XMcm7 from the QH3a fraction co-precipitated all of the XMcm2, XMcm4, and XMcm6 (Fig. 3G), suggesting that they form a single complex. Coomassie staining of the QH3a fraction (Fig. 3E, lane 2) showed that the complex is essentially pure at this stage, containing only XMcm2, -4, -6, and -7. Densitometry of the Coomassie-stained bands separated further on a 6% gel gave the ratio of XMcm2:XMcm4:XMcm6:XMcm7 as 1:0:1:1:1.1:1.1, suggesting that this complex is a tetramer of XMcm(2 + 4 + 6 + 7).

Gel filtration of the QH3b fraction showed XMcm4, -6, and -7 co-migrating with an apparent molecular mass of ~600 kDa (Fig. 3D). Immunoprecipitation of XMcm7 from the QH3b fraction co-precipitated all of the XMcm4 and XMcm6 (Fig. 3G), suggesting that these proteins form a single complex. Further purification of QH3b to apparent homogeneity by hydrophobic chromatography yielded approximately equal quantities of XMcm4, -6, and -7 as judged by Coomassie staining (Fig. 3E, lane 3). Using the QH3a fraction as a standard, quantitative Western blotting of the QH3b fraction gave the ratio of XMcm4:XMcm6:XMcm7 as 1:0:1:0:1.1. Together with the gel filtration and immunoprecipitation results, this suggests that the complex largely comprises a XMcm(4 + 6 + 7)2 hexamer. However we cannot rule out the presence of minor forms that deviate slightly from this stoichiometry, such as XMcm(4 + 6 + 7 + 7 + 7).

Because the immunoprecipitation experiment shown in Fig. 1C suggested that XMcm2 can be separated from XMcm7 by treatment with high salt, we performed gel filtration on the QH3a fraction in 1 M KCl (Fig. 4A). Most of the XMcm2 was displaced from the XMcm(2 + 4 + 6 + 7) complex, while the XMcm4, -6, and -7 that had separated from XMcm2 shifted from an apparent molecular mass of ~500 kDa (Fig. 4A, fraction 5) up to an apparent molecular mass of ~600 kDa (Fig. 4A, fraction 3), keeping an approximately constant stoichiometry. When this high molecular mass fraction was gel filtered again in low salt (Fig. 4B), its migration at ~600 kDa was unchanged (XMcm4, -6, and -7 peaking at fraction 3 and 4). This pattern was identical to the behavior of the XMcm(4 + 6 + 7)2 hexamer (Fig. 3D), suggesting that upon removal of XMcm2 from XMcm(2 + 4 + 6 + 7), the XMcm(4 + 6 + 7)2 hexamer was

**Fig. 1.** MCM/P1 proteins in Xenopus egg extracts are predominantly in the form of heterohexamers. A and B, crude Xenopus extracts were fractionated by gel filtration (A) or glycerol gradient sedimentation (B). Fractions were analyzed by PAGE, immunoblotted, and probed with all six anti-MCM/P1 antibodies. Molecular mass markers (in kDa) are shown above. C, crude Xenopus extract was immunoprecipitated (I.P.) with antibodies against XMcm3 or XMcm7. Samples were immunoblotted with all six anti-MCM/P1 antibodies. start, starting extract; s/n, unpurified proteins; 0.3M wash and 1M wash, proteins remaining bound to antibody after washing in 0.3 M and 1 M KCl.
reformed. Consistent with this interpretation, when XMcm7 immunoprecipitates were washed in 1 M salt, XMcm4 and -6 remained tightly associated (Fig. 1C and data not shown). In contrast, the XMcm2 displaced from the XMcm(2 + 4 + 6 + 7) complex shifted down to an apparent molecular mass of ~270 kDa under high salt gel filtration (Fig. 4A, fraction 9) and remained there when gel-filtered again in low salt (Fig. 4D). Given that the starting material for the gel filtration was essentially purified XMcm(2 + 4 + 6 + 7) (Fig. 3E, lane 2), this suggests that the ~270-kDa XMcm2 complex is likely to be an elongated homodimer of XMcm2. The QH3a that continued to suggest that theXMcm(3–5) 4 + 6 + 7) complexes. Some of the more slowly migrating XMcm, XMcm4, -6, and -7, seen in 1 M salt might also represent an XMcm(4 + 6 + 7) trimer. Formation of these subcomplexes is also consistent with the immunoprecipitation data (Fig. 1C), which showed a tight interaction between XMcm3 and -5 and between XMcm4, -6, and -7, with XMcm2 forming a weaker interaction with the latter. In summary, we observe a similar, although not identical, set of complexes by chromatography and by high salt treatment, suggesting that these are components of a general disassembly pattern.

**Heterohexameric Complexes Can Be Reformed from the Subcomplexes**—There are two possible explanations for the ability of the subcomplexes to reconstitute RLF-M activity when mixed together (Fig. 2). One possibility is that the licensing activity defined as "RLF-M" (15, 19) in fact comprises several subcomplexes that each act separately on chromatin. Alternatively, when the different subcomplexes are mixed, they may reconstitute the XMcm(2–7) heterohexamer, which alone is responsible for providing RLF-M activity. We therefore determined whether heterohexamers could reassemble from mixtures of the subcomplexes (Fig. 6). When XMcm(3 + 5) was mixed with XMcm(2 + 4 + 6 + 7) and subjected to gel filtration, all six MCM/P1 proteins now appeared as a single peak with a molecular mass of 500–600 kDa, similar to the heterohexamer (Fig. 6A, left panel). Reconstitution of the XMcm(2–7) heterohexamer was confirmed by the co-precipitation of XMcm2 and -7 with anti-XMcm3 antibody (Fig. 6A, right panel). We conclude that mixtures of subcomplexes capable of providing RLF-M activity are capable of forming heterohexamers.

We then mixed the XMcm2 and XMcm(4 + 6 + 7), complexes that had been obtained by separation of XMcm(2 + 4 + 6 + 7) in 1 M KCl, as shown in Fig. 4. On mixing, the XMcm2 now migrated on gel filtration as expected as a component of the XMcm(2 + 4 + 6 + 7) complex (Fig. 6B, left panel). Consistent with this, XMcm2 and -7 were co-precipitated from the mixture in low salt, but most of the XMcm2 was removed in 1 M salt (Fig. 6B, right panel). We next investigated whether the complexes could associate promiscuously or whether they only as-

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**FIG. 2. Fractionation of MCM/P1 proteins by Q Sepharose and heparin chromatography.** A, crude RLF-M was applied to a Q Sepharose column and eluted with a KCl concentration gradient. Individual fractions were immunoblotted with all six anti-MCM/P1 antibodies. B, Q1, Q2, and Q3 fractions from the Q Sepharose column were pooled as indicated and assayed for RLF-M activity. C, Q1, Q2, and Q3 fractions were applied to a heparin column and eluted with a KCl concentration gradient. Fractions were immunoblotted with relevant anti-MCM/P1 antibodies. D, QH1, QH3a, and QH3b fractions from the heparin column were pooled as indicated and assayed for RLF-M activity.
associate in a specific order. When XMcm2 was mixed with XMcm(3\textsuperscript{5}), or when XMcm(4\textsuperscript{1} + 6\textsuperscript{1})\textsubscript{2} was mixed with XMcm(3\textsuperscript{5}), no complex formation was observed, either by gel filtration (Fig. 6, C and D) or by co-immunoprecipitation (data not shown). This suggests that there is an obligatory sequence to the assembly of the heterohexamer, involving first an interaction between XMcm2 and XMcm(4\textsuperscript{1} + 6\textsuperscript{1})\textsubscript{2} to form XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2}, followed by an interaction between XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2} and XMcm(3\textsuperscript{5}) to form the XMcm(2–7) heterohexamer.

Finally, we investigated whether the XMcm(3\textsuperscript{7}) dimer found in the Q2 and QH2 fractions could interact with XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2} in an analogous manner to the XMcm(3\textsuperscript{5}) dimer. When XMcm(3\textsuperscript{7}) was mixed with XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2} and then gel-filtered, all of the XMcm3 and -7 shifted up to a hexameric position, consistent with the formation of an XMcm(2\textsuperscript{3} + 4\textsuperscript{6} + 7\textsuperscript{7}) hexamer (Fig. 6E). This suggests that the XMcm(3\textsuperscript{7}) dimer is generated from XMcm(2\textsuperscript{3} + 4\textsuperscript{6} + 7\textsuperscript{7}) present in the extract, which does not provide RLF-M activity (Fig. 2).

Active RLF-M Is a Heterohexamer Containing All Six MCM/P1 Proteins—We have shown that mixtures of XMcm(3\textsuperscript{5}) and XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2} reassemble into heterohexamers (Fig. 6) and provide RLF-M activity (Fig. 2). We next wanted to determine whether heterohexamer assembly must take place before interaction with chromatin, or whether RLF-M activity can be built up on chromatin from individual subcomplexes. Fig. 7 shows that subcomplexes can bind separately to chromatin. Demembranated sperm nuclei were incubated in XMcm-depleted extract supplemented with combinations of Q1 (containing XMcm(3\textsuperscript{5})) or Q3 (containing XMcm(2\textsuperscript{4} + 6\textsuperscript{1})\textsubscript{2}). Chromatin was then isolated and immunoblotted for XMcm3 and -7 (Fig. 7A). This showed that both groups of subcomplexes bound separately to the chromatin at levels comparable with native RLF-M.

Previous work has shown that unlicensed chromatin can be assembled in extracts treated with the kinase inhibitor 6-DMAP (40); subsequent licensing of this 6-DMAP chromatin...
requires both RLF-B and RLF-M components of the licensing system (15, 16). Fig. 7B shows the effect of RLF-B on the ability of different MCM/P1 subcomplexes to be assembled onto 6-DMAP chromatin. The XMcm(2–7) complex in native RLF-M was only assembled onto chromatin in the presence of RLF-B (Fig. 7B, lanes 7 and 8). In contrast, both XMcm(3 + 5) and XMcm(2 + 4 + 6 + 7) bound to chromatin in the absence of RLF-B (Fig. 8B, lanes 4 and 5), while the addition of RLF-B actually decreased their binding (Fig. 7B, lanes 1 and 2). When XMcm(3 + 5) and XMcm(2 + 4 + 6 + 7) were mixed together, the quantity of XMcm3 and -7 bound to chromatin in the presence of RLF-B increased (Fig. 7B, lane 6), while in the absence of RLF-B it decreased (Fig. 7B, lane 6). This is consistent with the reformation of the active XMcm(2–7) complex (Fig. 6A), whose binding to chromatin is RLF-B-dependent. Taken as a whole, these results suggest that the chromatin binding of the separate complexes is illegitimate and cannot license the chromatin for replication.

To test this hypothesis, we performed a two-step licensing reaction (Fig. 8). XMcm-depleted extract was first supplemented with either XMcm(3 + 5) or XMcm(2 + 4 + 6 + 7) or both together. Sperm chromatin was then incubated in these extracts, under conditions similar to those used in Fig. 7A. After 15 min, chromatin was isolated, and a second incubation with complementary subcomplexes was performed. Fig. 8 shows that only chromatin incubated simultaneously with both XMcm(3 + 5) and XMcm(2 + 4 + 6 + 7) became licensed. Sequential treatment with the two individual subcomplexes gave only background levels of licensing. This suggests that the chromatin binding of the individual subcomplexes shown in Fig. 7 is unproductive and that the MCM/P1 proteins can only provide RLF-M activity when assembled into the XMcm(2–7) heterohexamer.

**DISCUSSION**

Subunit Composition of Different MCM/P1 Subcomplexes—In this paper, we have characterized the composition and function of MCM/P1 complexes present in Xenopus egg extracts. Since similar complexes are found in a range of other organisms, our conclusions are likely to apply to all eukaryotes. Using a combination of gel filtration, glycerol gradient sedimentation, and co-immunoprecipitation, we show that all six Xenopus MCM/P1 proteins are present mainly in the form of XMcm(2–7) heterohexamers, consistent with previous reports (18, 19, 27, 46). A small proportion (~10% of the total) appears to be in the form of an XMcm(2 + 3 + 4 + 6 + 7 + 7) heterohexamer. Previous results showing an anomalously slow sedimentation...
value (19, 47) appear to be due to the instability of these complexes.

Fractionation of extracts by a number of different techniques readily generated a reproducible set of subcomplexes. We have characterized these complexes and provide evidence that they consist mainly of an XMcm(3 + 5) dimer, an XMcm(3 + 7) dimer, an XMcm2 dimer, an XMcm(2 + 4 + 6 + 7) tetramer, and an XMcm(4 + 6 + 7) hexamer. These are the predominant combinations, although minor variations in subcomplex composition may occur. For example, a small proportion of XMcm3 was identified that was not associated with the other MCM/P1 proteins. Further, slight deviations of the relative abundance of XMcm4, -6, and -7 in the QH3b fractions (which vary from preparation to preparation) may suggest the presence of complexes that deviate slightly from the 2:2:2 composition that we propose. However, these minor variations do not affect the major conclusions that we draw. Although this is the first comprehensive analysis to be performed, complexes consistent with our proposals have been observed in other eukaryotes. An XMcm(3 + 5) dimer has been identified as the major form of these two proteins in human cell extracts (32–35), while a tight association between XMcm3 and -5 has been observed in S. cerevisiae (48) and S. pombe (37). In mammalian cell extracts, XMcm2, -4, -6, and -7 are predominantly found associated with one another but substantially free of XMcm3 and -5 (12, 32, 34–36, 38), consistent with our XMcm(2 + 4 + 6 + 7) complex. In mammalian extracts, further fractionation could separate XMcm2 from this complex, leaving XMcm4, -6, and -7 migrating on gel filtration as an apparent hexamer (12, 36), while in S. pombe, XMcm2 was shown to interact weakly with a complex containing XMcm4 and -6 (39), consistent with our generation of XMcm(4 + 6 + 7) from XMcm(2 + 4 + 6 + 7). Further, there is evidence of a hexameric complex lacking XMcm5 in Drosophila (31), consistent with our XMcm(4 + 6 + 7) complex. In S. pombe, XMcm2 was shown to interact weakly with a complex containing XMcm4 and -6 (39). S. pombe XMcm2 was also shown to interact with itself in a two-hybrid screen (39), consistent with our proposed XMcm2 homodimer. The separation of MCM/P1 heterohexamers into specific subcomplexes therefore appears to have been highly conserved throughout evolution, suggesting that this has functional importance.

A Sequential Assembly Pathway for MCM/P1 Proteins—One obvious explanation for the presence of distinct subcomplexes is that they are intermediates in the assembly pathway for the heterohexamer. As summarized in Fig. 9, we show here for the first time that the subcomplexes can combine to reform the heterohexamer and that they do this only in a defined order. XMcm4, -6, and -7 are first assembled into a stable hexamer, mainly comprising XMcm(4 + 6 + 7) hexamer. XMcm2, probably in the form of a homodimer, can then associate with the XMcm(4 + 6 + 7) hexamer to form two XMcm(2 + 4 + 6 + 7) tetramers. A heterodimer of XMcm3(3 + 5) can then interact with these complexes to form the XMcm(2–7) heterohexamer. A small proportion (~10%) of XMcm3 and -7 present as an XMcm(3 + 7) dimer can also interact with the XMcm(2 + 4 + 6 + 7) tetramer to form an XMcm(2 + 3 + 4 + 6 + 7 + 7) hexamer, which does not contribute to RLF-M activity. All of the different subcomplexes are likely to be in equilibrium with one another, and the scheme shown in Fig. 9 appears to be both an assembly and a disassembly pathway. Although there is no direct evidence for disassembly occurring in vivo, Kubota et al. (18) have described the preferential release of XMcm3 and -5 from nuclei when replication forks are stalled with aphidicolin.

The assembly of precursor complexes as intermediates in multiprotein complex assembly was first noted for hemoglobin, where β-globin can associate into homotetramers before being assembled into the active α2β2 tetramer (49). The assembly pathway for small nuclear ribonucleoprotein complexes has striking similarity to the one we propose here. The small nuclear ribonucleoprotein core proteins eventually form a heterohexamer (50) but first assemble into a hexamer of two identical trimers, one of which is replaced by two dimers of other core proteins (51). As with the small nuclear ribonucleoprotein core proteins, the close similarity between all members of the MCM/P1 family may necessitate a defined assembly pathway involving high molecular weight intermediates.

In Xenopus eggs, the equilibrium between the different subcomplexes appears to favor formation of the heterohexamer, since most of the MCM/P1 proteins in crude extract can be co-immunoprecipitated (Refs. 18, 19, 27, and 46; this paper). The heterohexamer may also predominate in the Drosophila early embryo and in S. pombe (30, 31). In contrast, in mammalian cells (33, 34, 36) and in S. cerevisiae (48, 52), the heterohexamer is apparently not abundant, with XMcm(2 + 4 + 6 + 7) and XMcm(3 + 5) being the predominant forms. The cause of this difference in relative abundance of the subcomplexes is unclear, but given the weak association between the subcomplexes and the ease with which the heterohexamer disassembles, additional co-factors may be required to maintain the heterohexamer. Indeed, there is evidence for heterohexamer assembly factors in Xenopus, since the reconstitution of RLF-M activity from subcomplexes can be enhanced by the addition of XMcm-depleted extract (Fig. 8).2 This heterohexamer assembly factor may plausibly be a chaperone-like protein that prevents illegitimate association between the subunits and favors formation of the heterohexamer. Given that only the heterohexamer can provide RLF-M licensing activity, regulation of such a heterohexamer assembly factor could provide a powerful way of regulating DNA replication, particularly in mammalian cells where heterohexamer assembly is apparently not favored.

**RLF-M Activity Can Only Be Provided by the Heterohexamer**—We show here that only mixtures of subcomplexes capable of reforming the XMcm(2–7) heterohexamer are capable of providing RLF-M activity. This is consistent with immunoprecipitation studies in Xenopus (18) and genetic studies in yeast, showing that each of the six MCM/P1 proteins is essen-

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2 T. A. Prokhorova, unpublished data.
occurred it interacts tightly with chromatin and resists elution.

A heterohexamer is highly salt-sensitive, but once licensing has loaded onto chromatin during the licensing reaction: the free mammalian cells has been reported to show weak helicase activity. Active MCM/P1 heterohexamer, we are now in a position to test whether individual subcomplexes cannot associate productively with chromatin.

We thank Angus Lamond, Tom Owen-Hughes, Neil Perkins for comments on the manuscript.

Acknowledgments—We thank Angus Lamond, Tom Owen-Hughes, Neil Perkins for comments on the manuscript.

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