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The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides

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Replication licensing factor (RLF) is involved in preventing re-replication of chromosomal DNA in a single cell cycle, and previously has been separated into two components termed RLF-M and RLF-B. Here we show that *Xenopus* RLF-M consists of all six members of the MCM/P1 protein family, XMcm2–XMcm7. The six MCM/P1 polypeptides co-eluted on glycerol gradients and gel filtration as complexes with a mol. wt of ~400 kDa. In crude *Xenopus* extract, all six MCM/P1 polypeptides co-precipitated with anti-XMcm3 antibody, although only XMcm5 quantitatively co-precipitated from purified RLF-M. Further fractionation separated RLF-M into two sub-components, one consisting of XMcms 3 and 5, the other consisting of XMcms 2, 4, 6 and 7. Neither of the sub-components provided RLF-M activity. Finally, we show that all six MCM/P1 proteins bind synchronously to chromatin before the onset of S-phase and are displaced as S-phase proceeds. These results strongly suggest that complexes containing all six MCM/P1 proteins are necessary for replication licensing.

Keywords: DNA replication/licensing factor/MCM/P1/RLF-M

Introduction

Replication licensing factor (RLF), 'licenses' replication origins during late mitosis or early interphase by putting them into an initiation-competent state (Blow and Laskey, 1988; Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995a). RLF is inactive during metaphase, and rapidly becomes activated on exit from metaphase when it can modify chromatin before nuclear envelope assembly is complete (Blow, 1993; Kubota and Takisawa, 1993; Mahbubani *et al.*, 1997). RLF activity cannot cross the nuclear envelope, so that once nuclear assembly is complete no further origins can become licensed (Blow and Laskey, 1988; Leno *et al.*, 1992; Blow, 1993; Coverley *et al.*, 1993). At the G_1–S phase transition, a second signal, S-phase promoting factor (SPF) (Blow and Nurse, 1990; Fang and Newport, 1991; Strausfeld *et al.*, 1994, 1996; Chevalier *et al.*, 1995; Jackson *et al.*, 1995), induces licensed origins within intact nuclei to initiate DNA replication, and in so doing removes or inactivates the licence. Thus in G_2, no active RLF remains in the nucleus, and the nuclear envelope must be permeabilized transiently to license the DNA and allow a further round of DNA replication. So long as the licensing signal and the initiation signal act sequentially, and can never act on DNA at the same time, the result will be the precise duplication of the DNA (Chong *et al.*, 1996).

Cell-free extracts from *Xenopus* eggs replicate chromosomal DNA under apparently normal cell cycle control. When protein kinase inhibitors such as 6-dimethylaminopurine (6-DMAP; Blow, 1993), staurosporine (Kubota and Takisawa, 1993) or olomoucine (Vesely *et al.*, 1994) are added to extracts in metaphase, they block the activation of RLF that normally takes place on exit from metaphase (Mahbubani *et al.*, 1997). Using *Xenopus* egg extracts treated with 6-DMAP, we have established an assay for the isolation of RLF (Blow, 1993; Chong *et al.*, 1995). By differential precipitation with polyethylene glycol (PEG), RLF can be separated into two components, RLF-M and RLF-B, both of which are essential to license the DNA for replication. Further purification of RLF-M revealed that it comprises a complex containing the *Xenopus* Mcm3 (XMcm3) protein, a member of the MCM/P1 protein family (Chong *et al.*, 1995). In an alternative approach, Kubota *et al.* (1995) characterized polypeptides present on licensed, but not unlicensed chromatin. One such protein was identified as XMcm3. Proteins co-immunoprecipitating with an anti-XMcm3 antibody showed a similar polypeptide pattern to the RLF-M complex (Chong *et al.*, 1995; Kubota *et al.*, 1995). Immunodepletion of XMcm2, XMcm3 and XMcm5 from *Xenopus* egg extract also inhibited the replication of nuclei prepared from G_2, but not G_1 cells, demonstrating the functional importance of the MCM/P1 proteins in licensing DNA replication (Madine *et al.*, 1995a).

Mcm (minichromosome maintenance) mutants were first isolated from *Saccharomyces cerevisiae* by their inability to replicate plasmids containing certain yeast replication origins (Maine *et al.*, 1984). Further analysis indicated that they are defective in the initiation of DNA replication. Homologous genes have been identified in a range of eukaryotes, to form the 'MCM/P1' family (Chong *et al.*, 1996). Sequence comparison of the genes suggest that they fall into six related groups termed *MCM2–MCM7*. All six MCM/P1 genes have now been cloned from *Xenopus* (Kubota *et al.*, 1995; Madine *et al.*, 1995a; Coué *et al.*, 1996; Romanowski *et al.*, 1996b; see accompanying manuscript).

Here we use specific antibodies against each of the individual *Xenopus* MCM/P1 proteins (see accompanying manuscript) to show that all six proteins are present in the purified RLF-M complex. Physical analysis of the

RLF-M complex suggests that the MCM/P1 polypeptides form at least two complexes of ~400 kDa. They all bind co-ordinately to chromatin early in the cell cycle and are released as a consequence of DNA replication.

Results

Purification of an active RLF-M complex containing all six MCM/P1 polypeptides

We have shown previously that the RLF-M complex from *Xenopus* contains XMcm3 and other MCM/P1 proteins (Chong *et al.*, 1995). Six different MCM/P1 genes have been cloned from *Xenopus* (Kubota *et al.*, 1995; Madine *et al.*, 1995a; Coué *et al.*, 1996; Romanowski *et al.*, 1996b; see accompanying manuscript), representing the six groups previously identified and termed *MCM2–MCM7* (Chong *et al.*, 1996). Using antibodies specific for each of the different MCM/P1 proteins (see Materials and methods), we followed RLF-M activity as well as the presence of each of the polypeptides during the chromatographic purification. In the first fractionation step, differential PEG precipitation, only ~50% of total XMcm2 and 3 protein was recovered in the RLF-M fraction. However, the supernatant from this step (containing the remaining XMcm2 and 3) had no RLF-M activity (data not shown). Co-fractionation of all six MCM/P1 proteins was observed on the subsequent Q-Sepharose, phenyl-Sepharose (not shown) and Superose 6 columns (Figure 1). The yield of RLF-M activity up to this stage is typically ~5%, losses being apparently due to exposure to high salt (data not shown). On the Superose 6 (gel filtration) column, all six MCM/P1 proteins co-eluted in the same fractions with an apparent mol. wt of 400–600 kDa (Figure 1C) and containing virtually all the RLF-M activity loaded onto the column (Figure 1A). The RLF-M complex is clearly visible on a Coomassie-stained gel of the fractions (Figure 1B). None of the contaminating proteins still present at this stage co-eluted with the MCM/P1 proteins or RLF-M activity.

The composition of the peak fraction on Superose 6 (fraction 27) is shown in Figure 2A. In comparison, we used anti-XMcm3 antibodies to immunoprecipitate XMcm3 and associated proteins from unfractionated *Xenopus* extracts (Figure 2B). The anti-XMcm3 antibodies co-precipitated all six MCM/P1 proteins from crude extract, removing the majority of each protein from the supernatants. This suggests that a significant proportion of the MCM/P1 proteins in the crude extract are physically associated with XMcm3, and that the anti-XMcm3 antibody does not disrupt these complexes. Coomassie staining of both purified RLF-M and the XMcm3 immunoprecipitate showed a similar series of polypeptides, suggesting that similar complexes are present in the two fractions (Figure 2A and B). However, when the purified RLF-M complex was immunoprecipitated using anti-XMcm3 antibodies, a different pattern of MCM/P1 proteins was observed (Figure 2C): XMcms 3 and 5 were removed quantitatively from the supernatant, whilst little XMcm4, 6 or 7 was co-precipitated with XMcm3, and only ~50% of the XMcm2. This suggests that some changes had occurred to the complexes during the RLF-M purification.

To confirm this result, we performed mobility shift experiments on a Superose 6 gel filtration column (Figure 3). XMcm3-specific antibodies were incubated with the RLF-M complex and applied to a gel filtration column (Figure 3B). This caused the XMcm3 protein to elute from this column at a higher molecular weight, indicating the binding of the antibody to this protein. In addition, nearly all of the XMcm5 protein was shifted as well, suggesting a tight interaction between these two proteins. However, the other MCM/P1 proteins did not show a change in behaviour, eluting exactly at the same position as the purified RLF-M complex (Figure 3A). In a similar experiment, purified RLF-M was immunodepleted using anti-XMcm3 antibody, and the supernatant was fractionated by gel filtration. Consistent with the results of the antibody shift experiments, the behaviour of XMcm6 and 7 on gel filtration was essentially unchanged following XMcm3 depletion (Figure 3C). These results suggest that purified RLF-M consists of at least two co-fractionating complexes of MCM/P1 polypeptides, one containing XMcm3 and 5, and the other containing XMcm2, 4, 6 and 7.

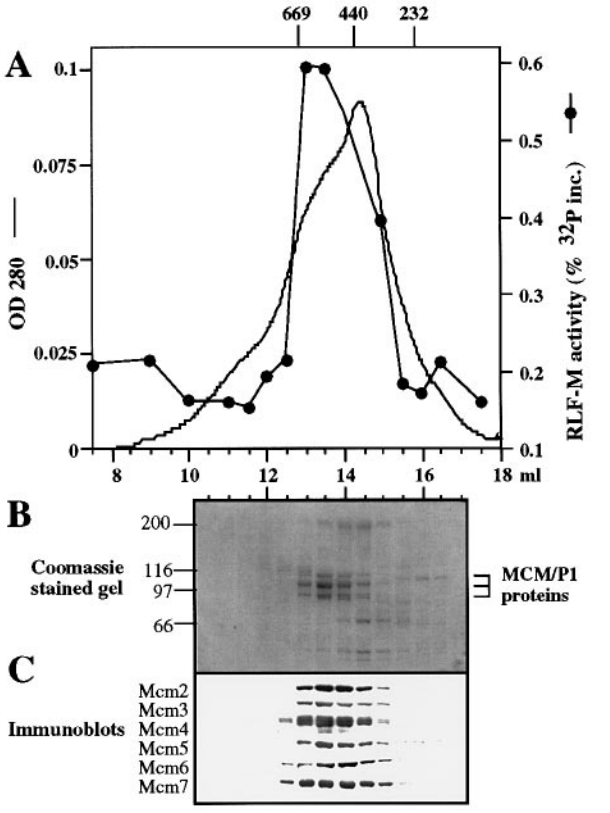

Fig. 1. All six MCM/P1 polypeptides co-elute with RLF-M activity on gel filtration. RLF-M was purified from interphase extract up to the phenyl-Sepharose step and was loaded onto a 24 ml Superose 6 column. (**A**) UV trace (——) and RLF-M activity (–●–). The migration of molecular weight markers (in kDa) is shown above. (B and C) Fractions were separated on 7.5% polyacrylamide gels and stained with Coomassie (**B**) or immunoblotted sequentially for the presence of each of the XMCM/P1 proteins (**C**). The migration of molecular weight markers (kDa) and the RLF-M proteins are indicated.

Physical properties of the RLF-M complex

We subjected purified RLF-M and unfractionated egg extract to both glycerol gradient centrifugation and gel filtration to study the physical properties of the MCM/P1 complex (Figure 4). All six MCM/P1 proteins from

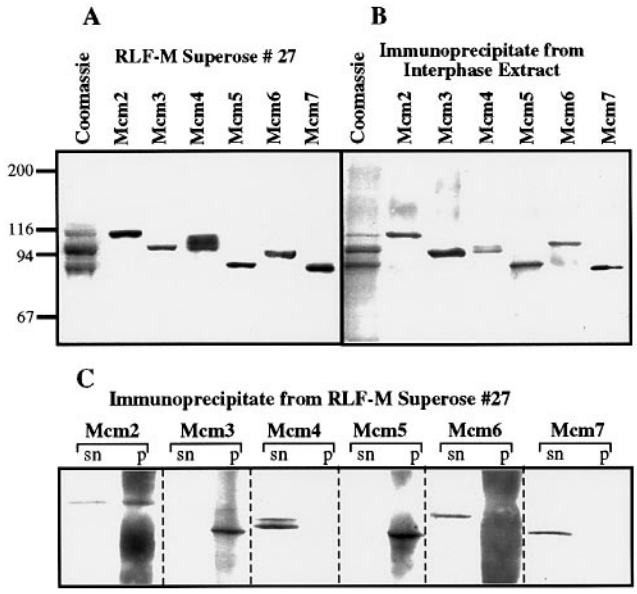

Fig. 2. All six polypeptides co-precipitate from crude extract but not from purified RLF-M. (A and B) The peak RLF-M fraction (#27) from the Superose 6 column (**A**) and immunoprecipitates of crude *Xenopus* extract collected with anti-XMcm3 antibodies (**B**) were separated on 7.5% polyacrylamide gels. The gels were stained with Coomassie or immunoblotted for the presence of each of the XMCM/P1 proteins. (**C**) Purified RLF-M from the Superose column was immunoprecipitated with anti-XMcm3 antibodies. Proportional samples of the immunoprecipitated pellets (p) and the depleted supernatants (sn) were separated in 7.5% polyacrylamide gels and immunoblotted for the presence of each of the XMCM/P1 proteins.

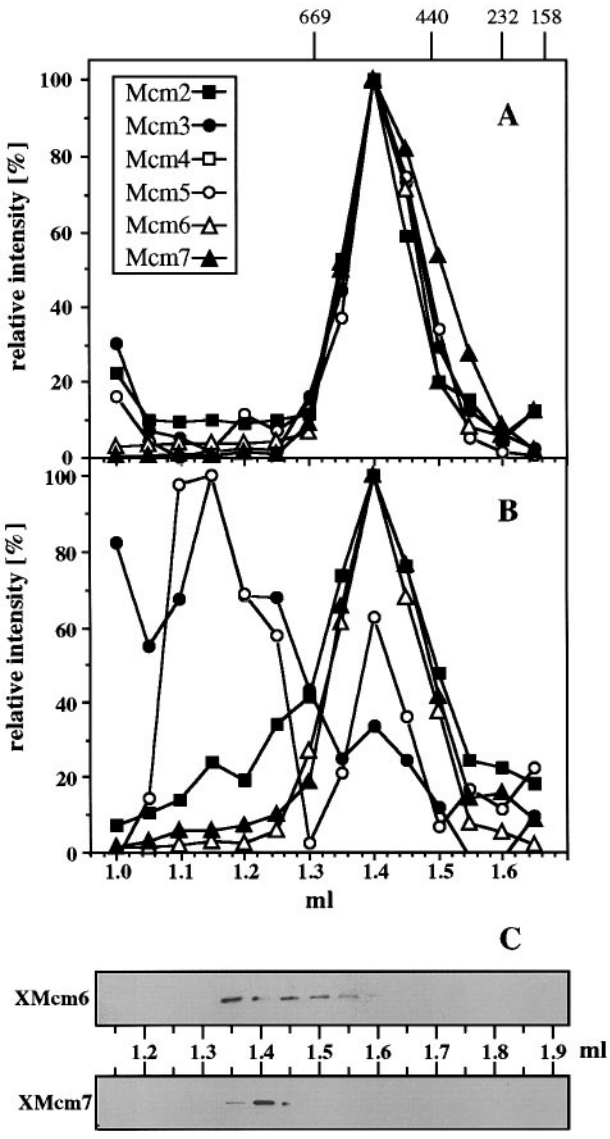

Fig. 3. XMcm3 and XMcm5 can be selectively shifted from purified RLF-M. The purified RLF-M complex from the Superose stage was incubated minus (**A**) or plus (**B**) XMcm3-specific antibodies and chromatographed on a 2.4 ml Superose 6 column. Fractions were subjected to PAGE and blotted for the presence of each of the XMCM/P1 proteins. The immunoblots were quantified and expressed as a percentage of the peak signal. –■– XMcm2, –●– XMcm3, –□– XMcm4, –○– XMcm5, –△– XMcm6, –▲– XMcm7. (**C**) Purified RLF-M was immunodepleted using the anti-XMcm3 antibody. The supernatant, containing no detectable XMcm3, was applied to a 2.4 ml Superpose 6 column and fractions were blotted for XMcm6 (upper panel) and XMcm7 (lower panel).

unfractionated extract sedimented on the glycerol gradient in a peak at ~13.5S (apparent mol. wt ~300 kDa; Figure 4A). In addition, a second smaller peak of XMcm2 and XMcm3 was observed, sedimenting at ~5S (apparent mol. wt 80 kDa). This low molecular weight material was separated away from RLF-M activity during purification (probably at the PEG precipitation step). Purified RLF-M gave a peak containing all six MCM/P1 proteins at ~13S, similar to crude extract, as well as a second peak (enriched for XMcms 4, 6 and 7) at 9S (apparent mol. wt 180 kDa; Figure 4B). On gel filtration, all six MCM/P1 proteins in both purified RLF-M and unfractionated extract eluted with an apparent mol. wt of ~550 kDa and a Stokes radius of 74 Å (Figure 4C and D). A proportion of the XMcm2 and 3 from unfractionated extract also eluted in a smaller peak at 48 Å (Figure 4C). The discrepancy between apparent molecular weights on glycerol gradient and gel filtration does not appear to be due to disruption of the complex in the glycerol gradient, since the glycerol gradient peak re-applied to gel filtration showed an unchanged migration of XMcm3 and 6 (Figure 4E). Instead, these results suggest that the MCM/P1 proteins are present in a complex with an extended shape and native mol. wt of between 300 and 550 kDa.

MCM/P1 sub-complexes

To achieve further chromatographic separation, the active RLF-M from the Superose column was subjected to ion exchange chromatography on MonoQ (Figure 5). As observed previously (Chong *et al.*, 1995, 1997), RLF-M activity eluted as a sharp peak (fraction 32) at ~330 mM KCl from this column (Figure 5A). Consistent with its behaviour on gel filtration, active RLF-M fractions contained all six MCM/P1 proteins (Figure 5C), apparently free of any other contaminating polypeptides (Figure 5B). This complex again migrated on gel filtration with an apparent mol. wt of 440–600 kDa (Table I, 'Prep. 1'). However, some of the XMcm3 and 5 also eluted at a lower salt concentration (Figure 5C) but had no associated RLF-M activity (Figure 5A). This early eluting XMcm3 and 5 migrated on gel filtration at an apparent mol. wt of 160–400 kDa, as though it had dissociated from the high molecular weight RLF-M complexes (Table I). The quantity of XMcm3 and 5 separating away from the main

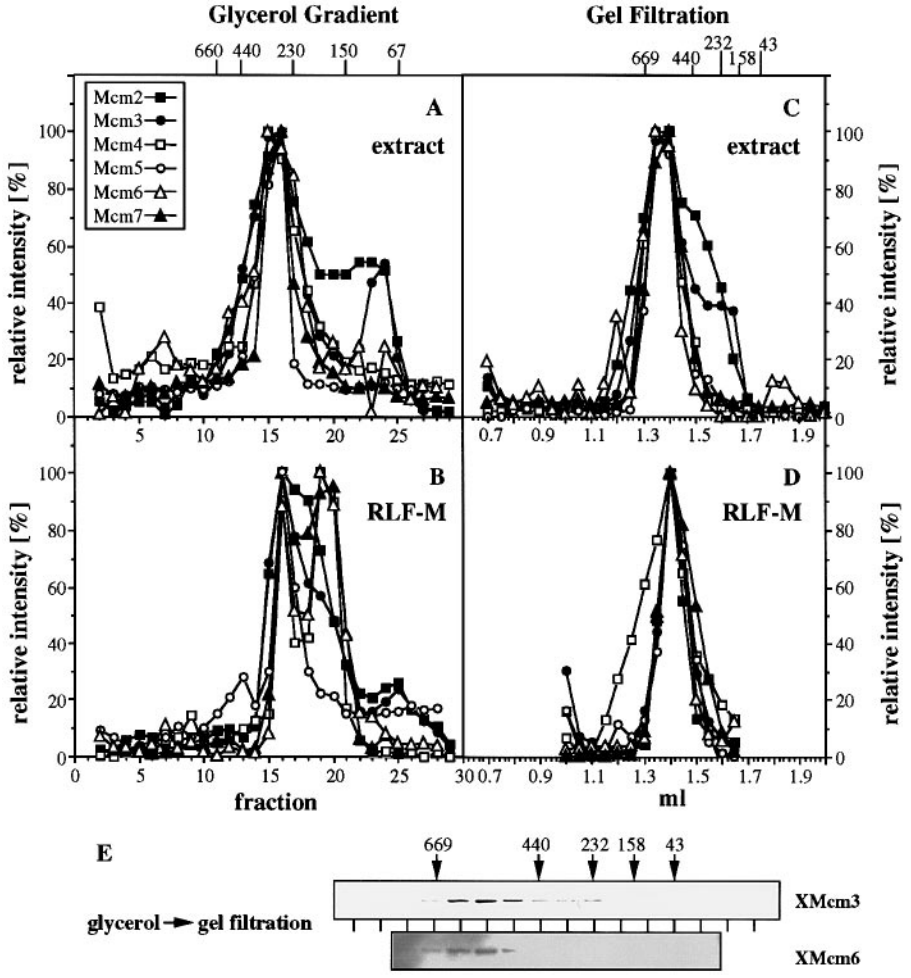

Fig. 4. The six MCM/P1 proteins are present in a high molecular weight complex. Diluted interphase extract (**A** and **C**) or purified RLF-M from the Superose stage (**B** and **D**) were analysed on 20–40% glycerol gradients (A and B) or on a 2.4 ml Superose 6 column (C and D). Column or gradient fractions were separated on 7.5% polyacrylamide gels and sequentially immunoblotted with antibodies against each of the XMCM/P1 proteins. The immunoblots were quantified and expressed as a percentage of the peak signal. –■– XMcm2, –●– XMcm3, –□– XMcm4, –○– XMcm5, –△– XMcm6, –▲– XMcm7. (**E**) Diluted interphase extract was fractionated on a 20–40% glycerol gradient, and the fast migrating peak of XMcm3 protein (apparent mol. wt 300–350 kDa) was separated on a 2.4 ml Superose 6 column. Fractions were blotted for XMcm3 (upper panel) and XMcm6 (lower panel).

peak varied considerably between different preparations (Table I). In some preparations, such as that shown by Chong *et al.* (1995), virtually all the XMcm3 and 5 applied to the MonoQ column eluted with the peak of RLF-M activity. In other preparations, such as 'Prep. 2' in Table I, virtually all the XMcm3 and 5 eluted early. Under these circumstances, when XMcm3 and 5 were separated quantitatively from the other MCM/P1 proteins, only very low RLF-M activity was associated with either peak (Table I). These results suggest that all of the MCM/P1 proteins are required for RLF-M activity.

Chromatin association of MCM/P1 polypeptides during the cell cycle

RLF proteins are expected to be bound to chromatin prior to the onset of S phase and to be displaced as replication occurs. We therefore examined the binding of all six MCM/P1 proteins to chromatin during the cell cycle *in vitro* (Figure 6). Demembranated sperm nuclei (which contain no MCM/P1 proteins) were incubated in *Xenopus* egg extracts. At the indicated times, chromatin was isolated and immunoblotted for the presence of all six MCM/P1 proteins. All six MCM/P1 polypeptides bound synchronously to chromatin, peak levels being reached after 20–30 min. DNA replication in these extracts started at ~40 min, and continued for a further 40 min (Figure 6, dashed line). During this period, the MCM/P1 proteins were removed from the chromatin, approximately in proportion to the extent of DNA replication. No significant difference in the kinetics of binding or displacement could be seen between the individual MCM/P1 proteins.

Discussion

RLF-M consists of all six MCM/P1 proteins

The MCM/P1 family consists of a closely related series of gene products found in a wide range of eukaryotes including insects, plants, amphibians and mammals (Chong *et al.*, 1996; Kearsey *et al.*, 1996). Sequence comparison of these MCM/P1 genes shows that all known genes cluster into six related groups, which have been named *MCM2–MCM7* (Chong *et al.*, 1996). All six MCM/P1 genes have now been cloned from human (Thömmes *et al.*, 1992; Hu *et al.*, 1993; Todorov *et al.*, 1994; Burkhart

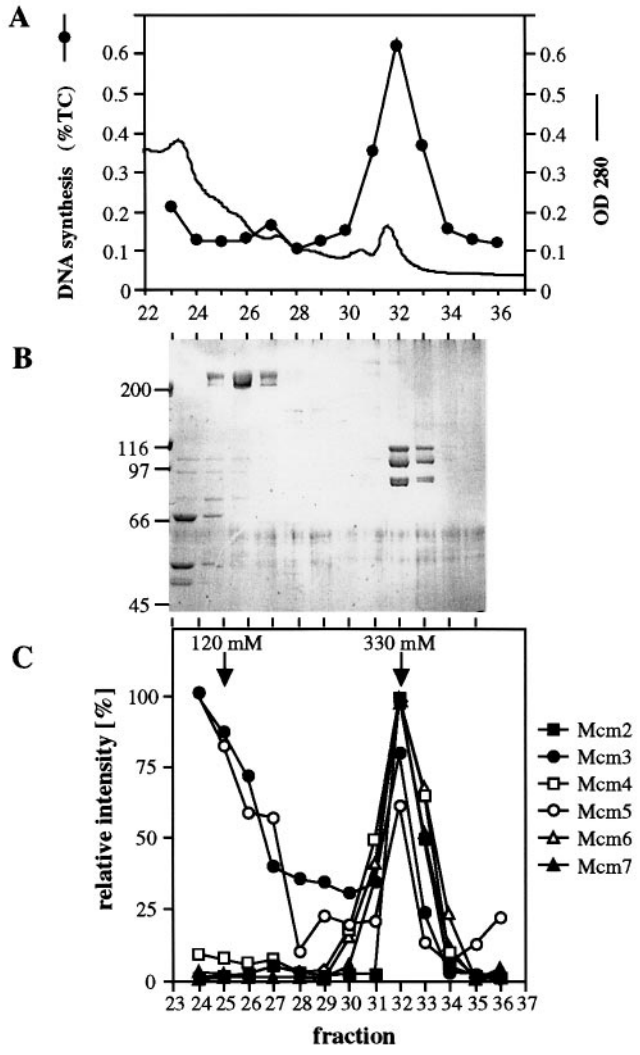

Fig. 5. A proportion of XMcm3 and XMcm5 separate from active RLF-M on MonoQ chromatography. The RLF-M complex from the Superose column was chromatographed on a 100 µl MonoQ column. (**A**) UV trace (——) and RLF-M activity (–●–) of eluted material. (**B**) Coomassie-stained gel of eluted protein. The migration of molecular weight markers (in kDa) is shown at the left. (**C**) Eluted fractions were immunoblotted for the presence of each of the XMCM/P1 proteins. The immunoblots were quantified and expressed as a percentage of the peak signal. –■– XMcm2, –●– XMcm3, –□– XMcm4, –○– XMcm5, –△– XMcm6, –▲– XMcm7.

Table I. Separation of MCM/P1 subcomplexes by MonoQ chromatography

Prep.	Peak (mM)	MCM/P1 present	RLF-M activity (%TC)	Apparent mol. wt on gel filtration
1	120	3–5	0.03	160–440
1	330	2–3–4–5–6–7	0.55	440–600
2	120	3–5	0.05	160–440
2	330	2–4–6–7	0.15	440–600

Early (120 mM) and late (330 mM) eluting fractions from the final MonoQ column in the purification of two different preparations of RLF-M were anlaysed for MCM/P1 protein composition, RLF-M activity and apparent molecular weight on gel filtration. Prep. 1 is the same as that shown in Figure 5.

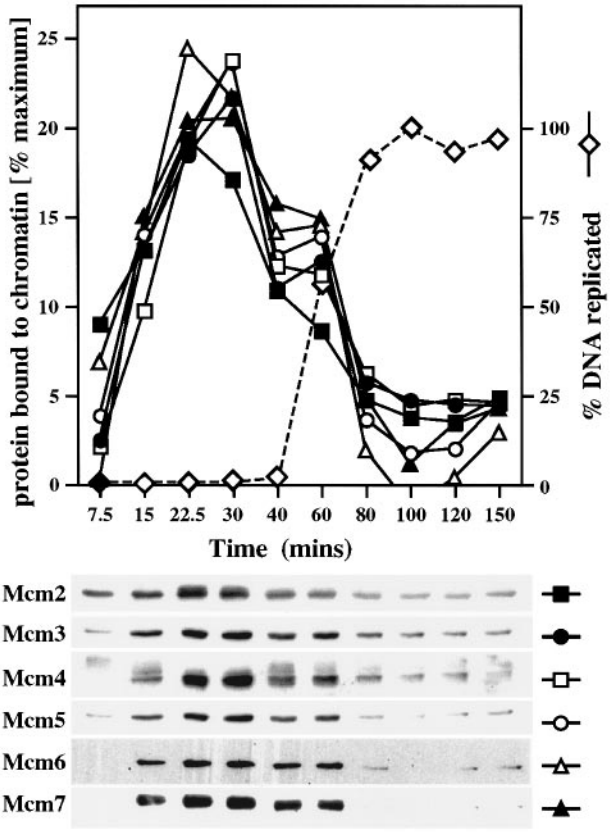

Fig. 6. All six MCM/P1 proteins bind synchronously to chromatin before S phase and are displaced as DNA replication proceeds. Demembranated *Xenopus* sperm nuclei were added to an interphase *Xenopus* egg extract and incubated at 23°C. Total DNA synthesis at different times was monitored by [α-^{32}P]dATP incorporation and expressed as the amount of DNA replicated at the respective time points (–◇–). Chromatin was isolated at different times and blotted for the presence of all six MCM/P1 proteins. The signal was quantified and expressed as a percentage of the peak signal. –■– XMcm2, –●– XMcm3, –□– XMcm4, –○– XMcm5, –△– XMcm6, –▲– XMcm7.

et al., 1995; Musahl *et al.*, 1995; Holthoff *et al.*, 1996; Schulte *et al.*, 1996) and *Xenopus* (Kubota *et al.*, 1995; Madine *et al.*, 1995a; Coué *et al.*, 1996; Romanowski *et al.*, 1996b; see accompanying manuscript). The development of antibodies specific for each of the six MCM/P1 proteins in *Xenopus* (Kubota *et al.*, accompanying manuscript) has allowed for the first time the analysis of all MCM/P1 proteins from a single eukaryotic organism.

In each of the chromatographic steps we found all six MCM/P1 proteins co-purifying with RLF-M activity. In particular, they co-eluted on gel filtration as a single active high molecular weight peak and were present in the most purified active RLF-M fraction. However, RLF-M activity was lost as soon as the MCM/P1 proteins were separated from each other, suggesting that all are required for RLF-M activity. This is consistent with the observation that despite their high degree of sequence conservation all MCM/P1 genes are essential for growth in yeast (reviewed in Tye, 1994; Chong *et al.*, 1996). Experiments in higher eukaryotes also suggest that each of the MCM/P1 proteins are indispensable. Immunodepletion of a *Xenopus* egg extract using antibodies against XMcm3 inhibited DNA replication (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995a), and all MCM/P1

proteins from *Xenopus* are needed to restore replication activity in these depleted extracts (Kubota *et al.*, accompanying manuscript). In human and mouse cells, microinjection of antibodies against HsMcm2 (BM28) (Todorov *et al.*, 1994, 1995) or MmMcm3 (Kimura *et al.*, 1994), or expression of antisense oligonucleotides against HsMcm7 (Fujita *et al.*, 1996) each inhibited the onset of subsequent DNA synthesis. *Drosophila MCM2* and *MCM4* genes also appear to be essential for mitotic DNA replication (Feger *et al.*, 1995; Treisman *et al.*, 1995). These results all strongly suggest that each of the six MCM/P1 genes are essential for DNA replication.

We show here by immunoblotting that all *Xenopus* MCM/P1 polypeptides bind to chromatin with similar kinetics, being loaded onto chromatin at exit from mitosis and then displaced as DNA replication proceeds. Similar results obtained by immunofluorescence are presented in the accompanying paper. Previous reports have shown a similar pattern of binding for XMcm2 (Madine *et al.*, 1995b), XMcm3 (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995a), XMcm4 (Coué *et al.*, 1996), XMcm5 (Madine *et al.*, 1995b) and XMcm7 (Romanowski *et al.*, 1996b). In mammalian tissue culture cells, Mcms 3, 4, 5 and 7 were displaced from chromatin, apparently being removed from the DNA as it replicated (Kimura *et al.*, 1994; Todorov *et al.*, 1995; Krude *et al.*, 1996). These observations are consistent with all six MCM/P1 proteins being part of the replication licensing system (see Chong *et al.*, 1996). Recently, the licensing of chromatin by RLF-M and RLF-B has been shown to be preceded by, and dependent on, the presence of the *Xenopus* origin recognition complex (XORC) on the chromatin (Rowles *et al.*, 1996). Similarly, the binding of MCM/P1 proteins to chromatin is also dependent on the *Xenopus* Cdc6 protein (Coleman *et al.*, 1996) as well as XORC (Coleman *et al.*, 1996; Romanowski *et al.*, 1996a; Rowles *et al.*, 1996). These results suggest the sequential loading during the cell cycle of proteins required for initiation in the vicinity of replication origins.

High molecular weight MCM/P1 complexes

On gel filtration of either unfractionated *Xenopus* extract or purified RLF-M, all six MCM/P1 proteins migrated with an apparent mol. wt of 440–600 kDa (Stokes radius of ~74 Å). However, on glycerol gradients, the majority of the proteins sedimented with an apparent mol. wt of ~300 kDa (13S). Glycerol gradient sedimentation and native gradient gel electrophoresis of MCM/P1 complexes in *Drosophila* gave results virtually identical to our results from *Xenopus* (Su *et al.*, 1996). The simplest explanation for the apparent discrepancy between gel filtration and glycerol gradient analysis is that the proteins are present in a complex with an elongated shape, increasing the apparent molecular weight on gel filtration and decreasing it on glycerol gradients. Assuming that the complex observed by glycerol gradient sedimentation is the same as that observed on gel filtration, a true molecular weight can be derived from a combination of these techniques using the relationship described by Siegel and Monty (1966). This suggests that the RLF-M complex has an apparent mol. wt of ~400 kDa, probably corresponding to a tetrameric complex of MCM/P1 proteins each with an average mol. wt of ~100 kDa. There must, therefore, be more than one type of tetrameric complex, since all six MCM/P1 polypeptides are present.

Other results also suggest the existence of different forms of MCM/P1 complex. Anti-XMcm3 antibodies co-precipitated only XMcm3 and 5 and a fraction of XMcm2 from purified RLF-M. Similarly, when purified RLF-M was pre-treated with anti-XMcm3 antibodies, only XMcm3 and 5 and a fraction of XMcm2 showed an apparent increase in molecular weight on gel filtration, leaving the migration of XMcm4, 6 and 7 virtually unchanged. XMcm3 and 5 also behave differently from the other MCM/P1 proteins on the final MonoQ fractionation step, as a proportion of XMcms 3 and 5 dissociated into a separate peak with a reduced molecular weight. Our observations on the purified RLF-M complex from *Xenopus* are in good agreement with observations on MCM/P1 complexes in human cells (Burkhart *et al.*, 1995; Musahl *et al.*, 1995; Schulte *et al.*, 1995, 1996). The human MCM/P1 proteins have an apparent mol. wt of 550 kDa on gel filtration. Upon treatment with 500 mM salt, individual complexes containing Mcm3/5 and Mcm4/6/7 sub-complexes can be identified. Anti-Mcm3 antibodies co-precipitated Mcm5 from human cells (Burkhart *et al.*, 1995), whilst anti-Mcm4 antibodies co-precipitated Mcm7, but not Mcm3 (Musahl *et al.*, 1995).

In contrast to the results obtained with purified RLF-M, immunoprecipitation of crude *Xenopus* extracts with anti-XMcm3 antibody co-precipitates all six MCM/P1 polypeptides (Kubota *et al.*, 1995; Madine *et al.*, 1995a,b; this study; accompanying manuscript). Despite this change in association, the behaviour of MCM/P1 proteins from crude extract on glycerol gradients and gel filtration is very similar to that observed with purified RLF-M. There appear to be two possible explanations for the change in co-association of MCM/P1 proteins before and after purification. One explanation is that MCM/P1 proteins in crude extract form several (tetrameric) complexes of differing subunit composition which re-arrange during the RLF-M purification. This redistribution of MCM/P1 proteins leads to a smaller number of preferential combinations such as Mcm3/5 and Mcm4/6/7. An alternative possibility is that in crude extract the (tetrameric) sub-complexes of MCM/P1 proteins interact to form larger complexes that can be immunoprecipitated intact but that are unstable during glycerol gradient and gel filtration analysis. This interpretation would be consistent with results in *Drosophila* where anti-Mcm5 antibodies co-precipitated Mcm4 only under low stringency conditions (no salt) but not in the presence of 150 mM KCl (Su *et al.*, 1996).

Whatever the exact composition of the native MCM/P1 complex, some degree of complex formation appears necessary for RLF-M function. When XMcms 3 and 5 are separated from XMcms 4, 6 and 7, RLF-M activity is lost. Activity cannot be restored by simply mixing the separate fractions (unpublished data), but they apparently must be returned to crude *Xenopus* extract to reconstitute activity (see accompanying manuscript). This suggests the presence of activities in crude extract which activate MCM/P1 polypeptides for RLF-M function. Identification of these other activities is likely to be important for understanding the biochemical function of the RLF-M complex.

Materials and methods

Preparation of chromatin

Chromatin for the licensing assay was prepared as described (Chong *et al.*, 1995, 1997). Briefly, demembranated sperm nuclei (at 100 ng DNA/μl) were assembled into chromatin for 12 min in a *Xenopus* extract activated with 0.3 mM $CaCl_2$ in the presence of 3 mM 6-DMAP. Chromatin was isolated by dilution in nuclear isolation buffer (50 mM KCl; 50 mM HEPES–KOH, pH 7.6; 5 mM $MgCl_2$; 5 mM EGTA; 2 mM β-mercaptoethanol; 0.5 mM spermidine; 0.15 mM spermine; 1 μg/ml each leupeptin, aprotinin and pepstatin) and centrifugation through a 15% sucrose cushion for 5 min at 6000 r.p.m. in a swing-out rotor. The chromatin pellet ('6-DMAP chromatin') was resuspended in nuclear isolation buffer at 80 ng DNA/μl and frozen in liquid nitrogen in 5 μl aliquots. For the analysis of chromatin-bound proteins, a similar protocol was used except that the nuclear isolation buffer was supplemented with 0.1% NP-40 and the chromatin pellet was resuspended in Laemmli loading buffer for analysis by SDS–PAGE (Chong *et al.*, 1995).

Purification of RLF-M

The purification of an active RLF-M complex was essentially as described (Chong *et al.*, 1995, 1997). Briefly, after the spin-crush step, activated egg extract (Blow, 1993) was diluted 5-fold in LFB1 [40 mM HEPES–KOH, pH 8.0; 20 mM K_2HPO_4/KH_2PO_4, pH 8.0; 2 mM $MgCl_2$; 1 mM EGTA; 2 mM dithiothreitol (DTT); 10% (w/v) sucrose; 1 μg/ml each of leupeptin, pepstatin and aprotinin] supplemented with 50 mM KCl (LFB1/50). Diluted extract was cleared by spinning at 50 000 *g* in a swing-out rotor, to generate 'licensing factor extract'. A 50% PEG 6000 solution in LFB1 was added to a final concentration of 4% PEG, incubated for 20 min on ice and proteins precipitated by spinning for 20 min at 10 000 *g* in a fixed-angle rotor. The pellet was resuspended in LFB1/50 (LFB1 plus 50 mM KCl) to a 5-fold concentration over neat extract and frozen for use as the crude RLF-B fraction. The supernatant was adjusted to 9.5% PEG and proteins were precipitated as before to generate the RLF-M fraction. The pellet was resuspended in 1 volume (with respect to undiluted egg extract) of LFB1/100 and adsorbed in batch onto an equal volume of Q-Sepharose (Pharmacia) equilibrated in LFB1/100. After washing, RLF-M activity was eluted with LFB1/325. The Q-Sepharose eluate was supplemented with 1 M solid KCl and applied in batch to an equal volume of phenyl-Sepharose (Pharmacia) equilibrated in LFB1/1000. After washing with LFB1/1000, activity was eluted with LFB1. Eluted protein was precipitated by addition of 0.5 volumes of 50% PEG in LFB1/50, incubated for 30 min on ice and spun for 10 min in a microfuge at 4°C. Pellets were resuspended in LFB1/75, insoluble particles removed by centrifugation in a microfuge for 10 min and the supernatant applied to a 24 ml Superose 6 column pre-equilibrated in LFB1/75 at 250 μl/min. Peak fractions of RLF-M activity (sizing from ~490 to 660 kDa) were pooled. These fractions were either used directly for analysis or applied to a 0.1 ml Mono Q column (SMART; Pharmacia) equilibrated in LFB1/100. Activity was eluted by a 20 column volume gradient from LFB1/100 to LFB1/500, and 100 μl fractions were collected, precipitated with 20% PEG and resuspended in 50 μl of LFB1/50. RLF-M activity eluted in a sharp peak over 1–2 fractions at ~330 mM KCl.

Licensing assay

RLF assays were performed as described (Chong *et al.*, 1995, 1997). PEG-precipitated RLF-B was diluted 10-fold in LFB1/50 supplemented with 2.5 mM Mg-ATP. One μl RLF-M fractions to be tested were incubated for 15 min at 23°C with 1 μl of diluted RLF-B and 0.3 μl of 6-DMAP chromatin (24 ng DNA). Then 5.7 μl of metaphase-arrested egg extract containing 3 mM 6-DMAP, 0.3 mM $CaCl_2$, 250 μg/ml cycloheximide, 25 mM phosphocreatine, 15 μg/ml creatine phosphokinase and 0.5 μCi [α-^{32}P]dATP was added. The reaction was stopped after 90 min at 23°C by addition of 200 μg/ml of proteinase K in 20 mM Tris–HCl, pH 7.5; 5 mM EDTA; 0.5% SDS. Total DNA synthesis was determined by trichloroacetic acid precipitation (Blow and Laskey, 1986; Chong *et al.*, 1997).

Antibodies

Rabbit polyclonal antibodies raised against each of the *Xenopus* MCM/P1 polypeptides were produced as described (see accompanying manuscript). We deduced their specificity for the different MCM/P1 polypeptides present in purified RLF-M as follows: (i) XMcm2 migrates on SDS–PAGE slower than any of the other MCM/P1 polypeptides; the anti-XMcm2 antibody recognized only this band, which was recognized by none of the other antibodies. (ii) XMcms 5 and 7 migrate together on SDS–PAGE ahead of the other MCM/P1 polypeptides; the anti-XMcm5 and anti-XMcm7 antibodies recognized only this band, which was recognized by none of the other antibodies. (iii) XMcms3, 4 and 6 migrate together on SDS–PAGE between the other MCM/P1 polypeptides; the anti-XMcm3, anti-XMcm4 and anti-XMcm7 antibodies recognized only this band, which was recognized by none of the other antibodies. (iv) In certain RLF-M preparations, XMcms3 and 5 quantitatively separate from the other four MCM/P1 polypeptides on the final MonoQ column (see Table I) and, in these preparations, no significant cross-reactivity between the antibodies was observed. (v) XMcm4 is phosphorylated quantitatively during metaphase (Coué *et al.*, 1996), moving away from XMcms3 and 6; under these conditions, no significant cross-reactivity between the antibodies was observed. Taken together, these results rule out the possibility of significant cross-reactivity of the antibodies to their non-cognate MCM/P1 polypeptides.

Immunoblots were performed according to Towbin *et al.* (1979) using PVDF membranes (Millipore) for immobilization of the proteins and the enhanced chemical luminescence technology (Amersham) for detection. Quantification of the blots was performed by laser densitometry using the ImageQuant software (Molecular Dynamics). For immunoprecipitation, antibodies were cross-linked to protein A–Sepharose (Pharmacia) as described (Harlow and Lane, 1988). Saturating amounts of the coupled beads were incubated for 1 h at room temperature with extract which had been cleared further by centrifuging for 1 h at 100 000 *g*. The precipitates were recovered by spinning in a microfuge, washed five times with 10 volumes of Tris-buffered saline and finally resuspended in two volumes of gel loading buffer.

Analytical gel filtration and mobility shift

Analytical gel filtration was performed on the SMART system (Pharmacia) using Superose 6, Superose 12 or Superdex 200 columns (all Pharmacia). Columns were run in LFB1/75 at 25 μl/min, and 50 μl fractions were collected. For mobility shift experiments, purified anti-XMcm3 IgG was incubated with purified RLF-M (Superose fraction) in a final volume of 50 μl for 1 h on ice before applying to a Superose 6 column on the SMART system. The column was run as above using LFB1/75 without DTT.

Four ml 20–40% glycerol gradients in LFB1/50 were pre-formed. Then 200 μl of cleared licensing factor extract or 200 μl of RLF-M (Superose fraction) were loaded on the top of the gradient and centrifuged for 17 h, at 58 000 r.p.m. in a SW60 rotor (Beckman) at 4°C. Markers were run in a parallel gradient. Fractions of 100 μl were taken from the bottom of the tubes.

Marker proteins for gel filtration and glycerol gradient centrifugation were: thyroglobulin (669 kDa; Stokes radius 85 Å); apoferritin (443 kDa, 57 Å, 17.6S); catalase (232 kDa, 52 Å, 11.3S); alcohol dehydrogenase (150 kDa, 46 Å, 7.4S); bovine serum albumin (66 kDa, 35 Å, 4.3S); ovalbumin (43 kDa, 3.6S) (all from Sigma).

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