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Licensing of DNA replication by a multi-protein complex of MCM/P1 proteins in *Xenopus* eggs

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In eukaryotes, chromosomal DNA is licensed for a single round of replication in each cell cycle. *Xenopus* MCM3 protein has been implicated in the licensing of replication in egg extract. We have cloned cDNAs encoding five immunologically distinct proteins associated with *Xenopus* MCM3 as members of the MCM/P1 family. Six *Xenopus* MCM proteins formed a physical complex in the egg extract, bound to unreplicated chromatin before the formation of nuclei, and apparently displaced from replicated chromatin. The requirement of six XMCM proteins for the replication activity of the egg extract before nuclear formation suggests that their re-association with replicated chromatin at the end of the mitotic cell cycle is a key step for the licensing of replication.

Keywords: MCM family/eukaryotic DNA replication/licensing of replication/*Xenopus* egg extract

Introduction

Two mechanisms operating during S- and G_2-phases safeguard the precise transmission of genetic information from a mother cell to daughter cells: the M-phase entry checkpoint-control and the licensing of replication. Both play a complementary role in ensuring that chromosomal DNA is exactly duplicated in each cell cycle. The M-phase entry checkpoint-control involves monitoring the presence of unreplicated DNA, so that the cell enters M-phase only after the completion of replication (Hartwell and Weinert, 1989). In contrast, the licensing of replication implies a mechanism which prevents the re-replication of DNA already replicated in the current S-phase (Blow and Laskey, 1988). The prevention of re-replication has been universally found in nuclear DNA at G_2 phase of the cell cycle (Rao and Johnson, 1970), and also in plasmid DNA injected into *Xenopus* eggs treated with cycloheximide to prevent entry into M-phase (Harland and Laskey, 1980). The latter finding led to a hypothesis that the control of re-replication is due to a *trans*-acting factor, which distinguishes replicated from unreplicated DNA, but not a *cis*-acting DNA element (Harland, 1981). The putative licensing factor is a positive *trans*-acting factor involved in this process (Blow and Laskey, 1988); it is required for the initiation of replication, and inactivated upon the replication of nuclear DNA. In addition, it is unable to cross the nuclear envelope. These features prevent the re-replication of nuclear DNA during S and G_2 phases, and re-replication is only licensed by passage through M-phase when the nuclear envelope is broken down to allow the access of active licensing factor to replicated DNA.

The MCM/P1 family of proteins, containing six known members (reviewed in Chong *et al.*, 1996; Kearsey *et al.*, 1996), was initially identified in a screen for genes involved in the initiation of replication of budding yeast *Saccharomyces cerevisiae*, and later proposed to be a candidate for a putative licensing factor (Tye, 1994). Five members of the MCM/P1 family of proteins have been identified in budding yeast, and all these gene products are essential for the initiation of replication. At G_1 phase of the cell cycle, all MCM proteins so far as tested with specific antibodies are present in the nucleus, and rapidly disappear from the nucleus upon the initiation of S-phase (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995). These features, their requirement for the replication, but their absence in replicated nuclei, is reminiscent of a putative licensing factor. However, the retention of apparent integrity of nuclear envelope throughout the mitotic cell cycle of budding yeast conflicts with the idea that the yeast MCM protein is a licensing factor. The MCM proteins cross the nuclear envelope, and accumulate in the nucleus at the end of M-phase.

An increasing number of MCM/P1 proteins have been identified in various eukaryotes and they are implicated in the replication of DNA (reviewed in Chong *et al.*, 1996; Kearsey *et al.*, 1996). In the fission yeast *Schizosaccharomyces pombe*, four genes have been isolated as members of the MCM/P1 family and six examples of MCM/P1 proteins have been identified in mammalian cells. In contrast to the budding yeast MCM proteins, fission yeast and mammalian MCM/P1 proteins are localized in the nucleus during the mitotic cell cycle (Thömmes *et al.*, 1992; Kimura *et al.*, 1994; Todorov *et al.*, 1994; Schulte *et al.*, 1995; Maiorano *et al.*, 1996; Okishio *et al.*, 1996), and the mammalian proteins have been found to be displaced from chromatin upon progression of replication (Kimura *et al.*, 1994; Todorov *et al.*, 1995; Fujita *et al.*, 1996; Krude *et al.*, 1996), thus suggesting the possible involvement of MCM/P1 proteins in the replication licensing phenomena of mammalian cells.

Using the *Xenopus* egg cell free system, *Xenopus* MCM3 (XMCM3) has been identified as a component of a putative licensing factor (Kubota *et al.*, 1995). By developing an assay system to measure the licensing activity of S-phase extract of *Xenopus* eggs (Kubota and Takisawa, 1993), we have identified the XMCM3-

containing protein complex as a candidate for the licensing factor. Independently of our work, Blow (1993) developed a similar assay for licensing activity in the S-phase egg extract, and successfully fractionated the licensing activity of the egg extract into two fractions, RLF-M and RLF-B (Chong *et al.*, 1995). RLF-M contains several proteins detected with an antibody against the conserved region of the MCM/P1 protein family, and one of the proteins has been identified as XMCM3 using a specific antibody. In contrast, RLF-B is apparently devoid of MCM proteins. Both fractions are required for licensing the replication of unreplicated as well as once-replicated chromatin. Two groups (Madine *et al.*, 1995a; Someya *et al.*, 1995) used antibodies against XMCM3 or conserved regions of the mammalian P1Cdc46 protein, to demonstrate that immunodepletion of these proteins resulted in a loss of replication activity of the egg extracts, and that protein fractions containing several putative MCM/P1 proteins could rescue the activity. In addition to the requirement of XMCM3 protein for replication activity of the egg extract, XMCM3 has been shown to behave as a licensing factor; it was associated with chromatin before the nuclear formation, dissociated from the chromatin upon progression of replication (Chong *et al.*, 1995; Kubota *et al.*, 1995), and it is required for the replication of permeabilized G_2 nuclei, but not for G_1 nuclei in the extract (Madine *et al.*, 1995a). Despite a controversy concerning the permeability of the nuclear envelope to the XMCM3 protein (Kubota *et al.*, 1995; Madine *et al.*, 1995a,b), all these reports suggest that XMCM3 protein is involved in the licensing activity of the egg extract.

Here, we have addressed three important questions that remained to be clarified. The first concerns the identity of proteins associated with XMCM3. The second concerns the role of such proteins in the licensing of replication. The third concerns the controversial ability of XMCM3 to cross the nuclear envelope. The present study shows that a physical complex of six MCM/P1 proteins plays an essential role in the licensing of replication observed in the egg extract.

Results

Protein composition of anti-XMCM3 immunoprecipitates from Xenopus egg extract

Using an anti-XMCM3 antibody, we have co-purified three major proteins, having apparent molecular masses of 112, 92 and 90 kDa, from the egg extract (Figure 1A, lane W). Stepwise elution of the immunoprecipitates further indicated that at least four distinct proteins were associated with XMCM3. Proteins eluted in high salt conditions (high salt eluate) consisted of three major proteins, p112, p100 and p90 (Figure 1A, lane 1). Subsequent elution in alkaline conditions (alkali eluate) apparently recovered most of the residual proteins bound to the precipitates, and produced two proteins p100 and p92 (Figure 1A, lane 2). In order to identify these proteins, polyclonal antibodies were raised against p112 and p92 proteins. Immunoblotting of the high salt and alkali eluates with anti-XMCM3 antibody, anti-p112 and anti-p92 antisera revealed that p112, p92 and p90 are immunologically distinct from XMCM3. Each antibody reacted with a single protein in these fractions. In addition, we

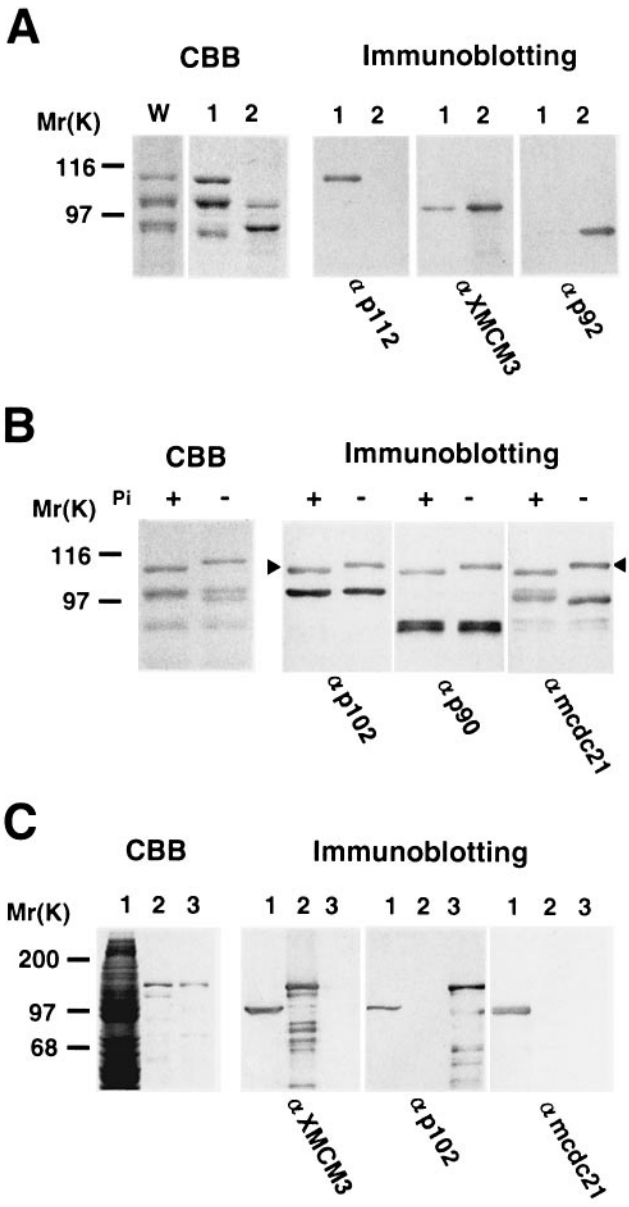

Fig. 1. Immunological identification of proteins associated with XMCM3 in the egg extract. (**A**) Dissociation of anti-XMCM3 immunoprecipitates into two components. The membrane-depleted S-phase egg extract was incubated with affinity purified anti-XMCM3 antibody-conjugated protein A beads, and the proteins recovered with the beads were eluted by high salt (0.8 M NaCl), followed by alkali (pH 11.0). Proteins bound to the anti-XMCM3 antibody (w), and those of the high salt (2.4 μg) (lanes 1) and alkaline eluates (2.2 μg) (lanes 2) were resolved by SDS–PAGE and visualized by Coomassie blue staining. Both eluates were immunoblotted with anti-p112 antisera, affinity purified anti-XMCM3 antibody and anti-p92 antisera. (**B**) Mobility shift of proteins in the high salt eluate upon phosphatase treatment. The high salt eluate of XMCM3 associated proteins (0.16 mg/ml) was treated with calf intestine alkaline phosphatase (500 U/ml) in the presence (+) and absence (–) of 20 mM sodium phosphate for 2 h at 20°C. Protein samples containing 1.6 μg of the high salt eluate were then resolved by SDS–PAGE and visualized by Coomassie blue staining, or immunoblotted with anti-p102, anti-p90 and anti-mouse cdc21 antisera, together with anti-p112 antisera. Immunoblotted bands of p112 are shown by arrowheads. (**C**) Specificity of antibodies against p100 proteins immunoprecipitated with anti-XMCM3 antibody. The membrane-depleted extract (0.6 μl)(lanes 1), GST-XMCM3 protein (1.9 μg) (lanes 2) and GST-XMCM6 protein (1.25 μg)(lanes 3) were resolved by SDS–PAGE and proteins were visualized by Coomassie blue staining. The same samples were processed for immunoblotting with anti-XMCM3 antibody, anti-p102 antisera, and anti-mouse cdc21 antisera.

found that the protein staining of p100 did not coincide with the immunostaining of XMCM3. The majority of the p100 protein was recovered in high salt eluate, but only a trace amount of XMCM3 was found in this fraction and most of XMCM3 was in alkaline eluate. These results suggest that p100 protein in the high salt eluate is different from XMCM3.

Dephosphorylation of proteins of the high salt eluate revealed that the p100 protein band consisted of two major distinct proteins. Figure 1B shows the gross mobility shifts in p112 and p100 proteins, but not p90 proteins, upon treatment with alkaline phosphatase. The p112 protein migrated at a lower mobility of 114 kDa, detected by both protein staining and immunostaining. The diffuse p100 protein band was separated into two distinct bands, one of 102 and the other of 98 kDa. Mobility shifts were completely blocked by phosphatase inhibitor, indicating that they are phosphoproteins. In contrast, proteins eluted at alkaline pH showed little mobility shift upon phosphatase treatment (data not shown).

The identity of proteins of the high salt eluate was investigated using specific antibodies against phosphatase treated proteins. Immunoblotting of the samples with anti-p102 and -p90 antisera revealed that both p102 and p90 are immunologically distinct from other proteins associated with XMCM3 (Figure 1B). Both antisera recognized a trace amount of these proteins in the alkali eluate (see Figure 6). Anti-mouse cdc21 antisera (prepared against the C-terminal region of mouse cdc21 protein, generously provided by H.Kimura) specifically recognized p98 protein generated upon phosphatase treatment, and the antisera recognized a broad band migrating at ~100 kDa in the presence of phosphatase inhibitor. These results indicate that six immunologically distinct proteins; p112, p102, XMCM3, p98, p92 and p90, are present in the immunoprecipitates.

The molecular structures of proteins associated with XMCM3

We have cloned the cDNAs encoding the five immunologically distinct proteins associated with XMCM3 by screening an expression cDNA library prepared from *Xenopus* oocyte mRNA with specific antibodies as probes, except for a putative mouse cdc21 homolog, p98. For p98, we have screened the library with mouse cdc21 cDNA as a probe. The sequencing of these clones revealed that all cDNAs contained a unique ORF (accession numbers of nucleotide sequences are U44047, U44050, U44049, U44048, and U44051 for cDNAs of p112, p102, p98, p92 and p90, respectively) and database comparison of these cDNA products revealed that they belong to the MCM/P1 family (see Table I). During the course of this study, essentially the same sequences for XMCM2 and 5 (Miyake *et al.*, 1996), and XMCM7 (Romanowski *et al.*, 1996), and a very similar sequence for XMCM4 (Xcdc21, 96.5% identical; Coué *et al.*, 1996) have been reported. Table I summarizes the relationship between the apparent molecular masses of proteins immunoprecipitated with anti-XMCM3 antibody and their identity as members of the MCM/P1 family. These results indicate that the five proteins associated with XMCM3 are five different members of the MCM/P1 family.

We further tested the identity of the cDNAs by expressing GST fusion proteins with these cDNAs. The criteria for the identification of XMCM3 associated proteins as MCM/P1 proteins are following. (i) The immunoblotting of egg extracts with each of the antisera was specifically blocked with corresponding GST fusion protein (data not shown). (ii) Affinity purified antibodies with corresponding GST fusion proteins, detected the same protein in the egg extracts and in the immunoprecipitates as the antisera did (compare Figures 1 and 6). (iii) Each GST fusion protein was specifically recognized by the corresponding antibody. The specificity of antibodies against p102, p98, and XMCM3 proteins to GST–XMCM3 and GST–XMCM6 proteins is shown in Figure 1C. In addition to the detection of a single protein in the egg extract, anti-p102 antisera detected GST–XMCM6, anti-XMCM3 antibody detected GST–XMCM3, and anti-mouse cdc21 antibody detected neither GST fusion protein. It should also be noted that the anti-p100 antisera previously used by us weakly cross-reacted with XMCM6 protein, though only at $<$500 fold dilution of the antisera. The reason why anti-p100 antisera exclusively detected XMCM3 may be the strong antigenicity of XMCM3 proteins compared with XMCM4 and XMCM6 proteins, both of which were presumably present in p100 protein bands of the chromatin fraction.

As to the mobility of bacterially produced proteins on SDS–PAGE, we found a distinct difference from immunoprecipitated XMCM2. Bacterially produced XMCM2 protein showed an apparent molecular mass of 114 kDa after removing the GST tag with thrombin, which is the same mobility observed with dephosphorylated p112 protein. This result is consistent with an apparent absence of specific kinases for XMCM2 protein in bacteria. As to p98, the antibody was raised against p100 proteins of high salt eluate and further purified against the C-terminal region of XMCM4 protein and tested for the immunoblotting of the egg extracts and the immunoprecipitates. The results obtained were essentially the same as those with anti-mouse cdc21 antisera except for the absence of minor cross-reacting proteins found with anti-mouse cdc21

Table I. XMCM3 and its associated proteins of the MCM/P1 family

Apparent molecular mass (kDa)	Dephosphorylated apparent molecular mass (kDa)	Gene name	Homologs (% identity)
112	114	XMCM2	ScMcm2 (49) BM28 (79)
100	100	XMCM3	ScMcm3 (43) HsP1 (70)
100	98	XMCM4	Spcdc21 (43) P1cdc21 (85)
92	92	XMCM5	ScCdc46 (51) P1Cdc46 (85)
100	102	XMCM6	Spmis5 (48) p105Mcm (72)
90	90	XMCM7	ScCdc47 (51) P1Cdc47 (83)

Homologs: this is the first description of the full-length of the DNA sequences for yeast and mammalian MCM/P1 proteins. Accession numbers are ScMCM2 (X53539), BM28 (X67334), ScMCM3 (X53540), HsP1 (X62153), Spcdc21 (X58824), P1cdc21 (X74794), ScCDC46 (U09242), P1CDC46 (X74795), Spmis5 (D31960), p105MCM (U46838), ScCDC47 (U14730) and P1CDC47 (D55716).

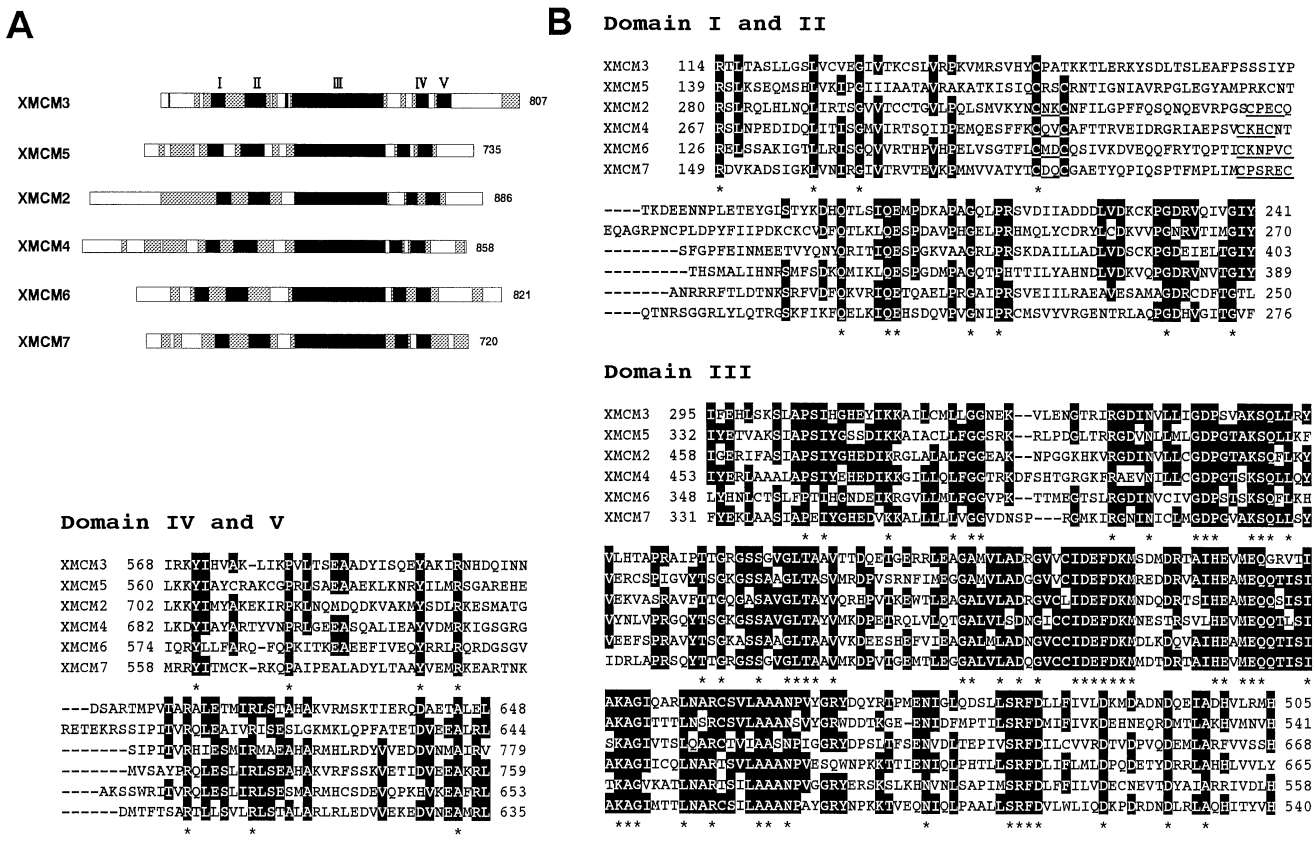

Fig. 2. *Xenopus* MCM/P1 protein family. (**A**) Scheme of the similarity among six *Xenopus* MCM proteins. Amino acid sequences of six *Xenopus* MCM proteins were predicted from the nucleotide sequences of six cDNAs cloned with specific antibodies against p112, p102, p100, p92 and p90 or with mouse cdc21 cDNA as probes. The lightly shaded regions indicate conserved domains of each member of the MCM/P1 family, and the closed regions indicate five putative conserved domains among MCM/P1 proteins. (**B**) Alignments of amino acid sequences of five conserved domains of XMCM proteins. Identical amino acids are boxed, and amino acids conserved between six XMCM proteins are indicated by stars.

antisera (cf. Figures 1B and 6C). As to p90, we reproducibly detected the presence of diffuse doublet bands, seen by protein staining as well as by immunostaining by anti-p90 antisera. We obtained one additional clone of XMCM7 (accession number, U66710), having the same length of polypeptide with sequence identity of >95% at the amino acid level. We therefore affinity purified the antisera with one of the GST–XMCM7 proteins. With the affinity purified antibody, we obtained the same results as with the anti-p90 antisera, indicating that both of the proteins recognized by the antibody are presumably XMCM7. At present, the exact reason why two protein bands were generated in the egg extract is not clear. Collectively, these results demonstrate that six *Xenopus* MCM/P1 proteins are co-precipitated with anti-XMCM3 antibody.

Figure 2A shows the schematic structures of the six XMCM proteins. Comparing the amino acid sequence of each member of the MCM/P1 proteins from yeast to human, we could identify fairly conserved regions of each member, shown as light shaded patterns. We further compared each conserved regions between MCM/P1 protein families. The homology search of these regions aided by the computer program GENETYX revealed five putative conserved regions among MCM/P1 proteins. Domain 3 is the most highly conserved region with a putative NTPase motif (Koonin, 1993), identical to domain 2 of the budding yeast MCM proteins (Tye, 1994). Domains 2 and 5 are essentially the same as previously reported MCM conserved domains 1 and 3 (Tye, 1994). In addition, we found two additional domains showing a good conservation between MCM/P1 families. Domains 1 and 4 possess fewer identical amino acids than other domains, but close examination of amino acid similarity suggests that they are also conserved between family and phyla. The gaps between domains 1 and 2, and domains 4 and 5 were assumed, since some MCM/P1 proteins showed no conservation of amino acids or the distance between these two domains.

Alignment of the amino acid sequences of these five domains of XMCM proteins revealed several unique motifs (Figure 2B). One is a Zn finger-like motif found at the end of domain 1 (underlined in the figure). In accordance with previous reports on yeast and mammalian MCM proteins (reviewed in Kearsey *et al.*, 1996), XMCM3 and 5 did not have this motif. We could determine the minimum sequence similarity of this motif between XMCM proteins: only the presence of four cysteine residues is conserved, but neither the amino acids nor the distances between cysteine residues is conserved. Moderately conserved domain 5 possesses a heptad R/K repeat, which is universally conserved in the MCM/P1 family of proteins. Besides the conserved regions among MCM/P1 proteins, we found alternately repeated clusters of basic and acidic amino acids in the N-terminus of XMCM2. A similar structural motif has been found in XMCM3 (Kubota *et al.*, 1995). Acidic amino acid clusters

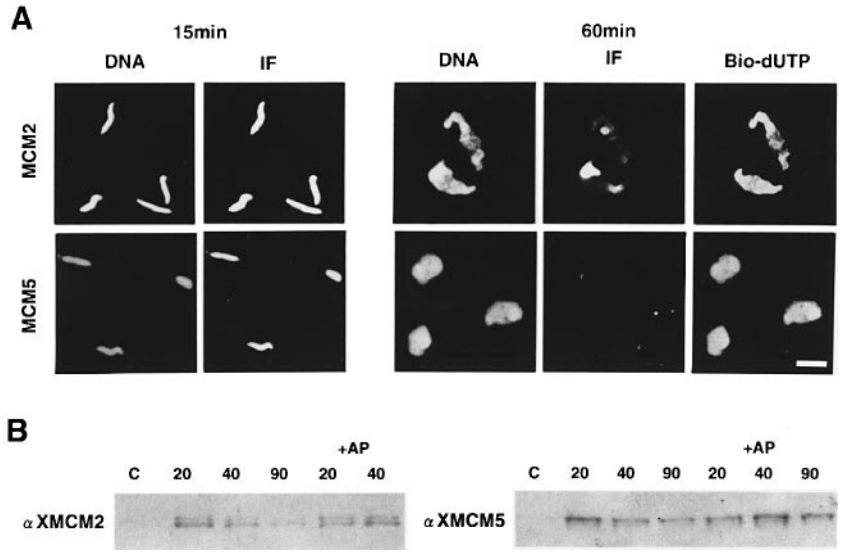

Fig. 3. Licensing behavior of XMCM proteins. (**A**) Immunofluorescence localization of XMCM2 and XMCM5 proteins. Sperm chromatin was incubated in the S-phase egg extract for 15 min and 60 min at 23°C. Samples taken at various times were fixed and processed for the immunofluorescent detection of XMCM2 and XMCM5 proteins. DNA was visualized with Hoechst 33258, and XMCM proteins (IF) were detected by mouse anti-XMCM2 and -XMCM5 antisera followed by FITC-labeled anti-mouse IgG. Replication activity was detected as the incorporation of biotin-dUTP into DNA followed by Texas Red–streptavidin. The fluorescence of each sample was directly photographed under the same settings. Scale bar, 20 μm. (**B**) Immunoblotting of XMCM2 and XMCM5 in the chromatin fractions. Sperm chromatin was incubated in the egg extracts with (+AP) and without 10 μg/ml aphidicolin for the indicated times at 23°C. Chromatin fractions were then prepared as described (Kubota *et al.*, 1995). The chromatin binding proteins were resolved by SDS–PAGE, and each sample was immunoblotted with anti-XMCM2 and anti-XMCM5 antisera. Control (c) was obtained by incubating the extract without sperm chromatin for 90 min at 23°C.

were also found in most of the MCM proteins, explaining why the mobility of each protein on SDS–PAGE was lower than the molecular mass calculated from the predicted amino acid sequence.

Licensing behavior of XMCM proteins in the egg extracts

The overall structural similarity between *Xenopus* MCM proteins coincided with their behavior during the cell cycle. Immunofluorescence localization of XMCM proteins during the cell cycle showed that they became associated with chromatin before the formation of nuclei, and were displaced from the nuclei upon progression of replication. Representative data for XMCM2 and XMCM5 are shown in Figure 3. The immunofluorescence signal of both XMCM2 and XMCM5 indicated a uniform distribution of these proteins on the decondensed chromatin before nuclear formation. After the formation of nuclei, upon progression of replication, most of the immunofluorescence signals disappeared from the replicating nuclei. Displacement of XMCM proteins from the replicating nuclei was further examined by quantifying the immunofluorescence intensity of each XMCM protein in the nucleus (Figure 4A). Decrease in the immunofluorescence intensity of each XMCM signal clearly coincided with increase in the incorporation of fluorescence labeled dCTP into the nucleus. Immunoblotting of the chromatin fraction further confirmed their association with chromatin before nuclear formation and their displacement from chromatin upon progression of replication. The relative amounts of XMCM3 and XMCM6 remaining on the chromatin as measured by Western blotting paralleled the intensity measured by immunofluorescence (Figure 4C; see also Figure 3B for XMCM2 and XMCM5; similar data for all six XMCM proteins were reported by Thömmes *et al.*, 1997).

The displacement of XMCM proteins from replicating nuclei depended on the replication activity of the extract, but not on M-phase-promoting (MPF) activity. Figure 5 shows that licensed chromatin, which had been obtained by incubating sperm chromatin in a membrane-depleted fraction of S-phase egg extract to load XMCM proteins onto the chromatin, became condensed in the M-phase egg extracts, but XMCM3 was still present on the condensed chromatin. However, without such pre-assembly of XMCM proteins, the sperm chromatin became condensed in the M-phase extract without any detectable signal of XMCM3 on the chromatin, though an equivalent amount of XMCM proteins was present in both egg extracts (Kubota *et al.*, 1995). These results indicate that additional factor(s) active in the S-phase, but not M-phase, extract are required for the association of XMCM proteins with chromatin, and that M-phase activity is sufficient to induce condensation of the chromatin, but not to dissociate XMCM3 proteins from the chromatin.

In S-phase extract, displacement of XMCM proteins from chromatin was inhibited by aphidicolin, at concentrations which completely inhibited the activity of DNA polymerase α in the egg extract (Kubota and Takisawa, 1993). The immunoblotting of chromatin fraction indicated that all six XMCM proteins remained on chromatin fractions in the presence of aphidicolin (see Figures 3B and 4C for XMCM2, 3, 5 and 6). In accordance with the immunoblotting data, the immunofluorescence signals of XMCM2, XMCM4, XMCM6 and XMCM7 persisted in the nucleus in the presence of aphidicolin (Figures 4B and 5). A similar behavior of *Xenopus* cdc21 homolog (XMCM4) has been recently reported with *Xenopus* eggs

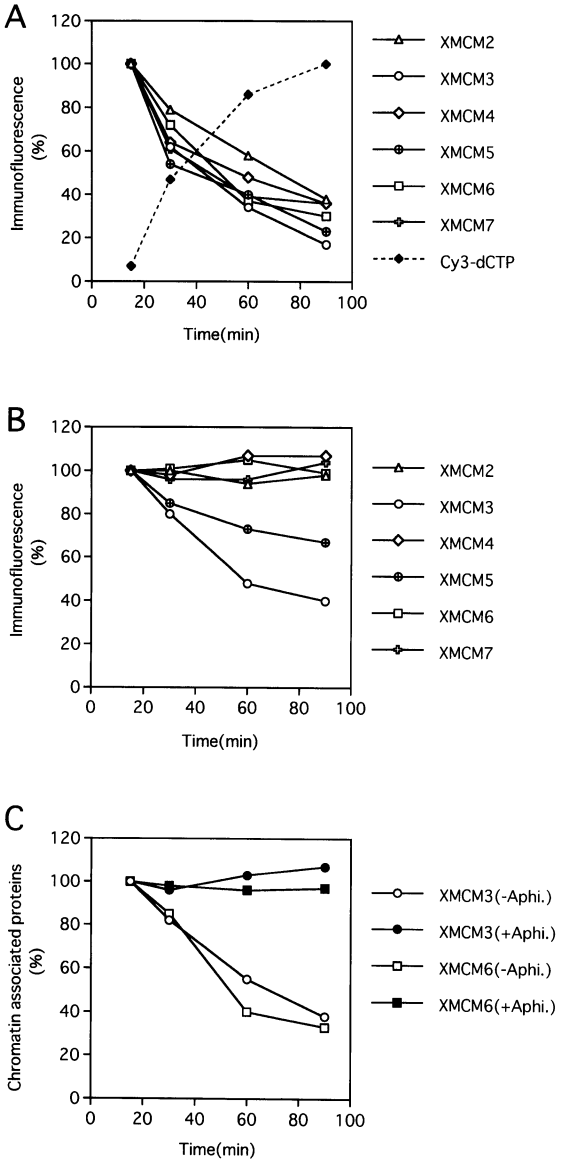

Fig. 4. Displacement of XMCM proteins from the nuclei upon progression of replication. Sperm chromatin was incubated in the S-phase extract in the absence (**A**) and presence (**B**) of 20 μg/ml aphidicolin for appropriate time indicated in the figure, and samples of 5 μl were taken and processed for observation of immunofluorescence of each XMCM proteins and Cy3-dCTP. The fluorescence intensities of ~20 nuclei were analyzed, and average intensities per nucleus were estimated. Relative average intensities were then plotted against time, taking the mean value at 15 min incubation as 100% for XMCM proteins, and at 90 min incubation as 100% for Cy3-dCTP incorporation. SEM was within 20% of each mean value. (**C**) Sperm chromatin was incubated in the S-phase extract, and samples of 25 μl were taken and processed for immunoblotting with anti-XMCM3 and XMCM6 antibody. The intensity of each immunoblotted band was quantitated, and relative values were plotted against incubation time, taking the value at 15 min incubation as 100%.

(Coué *et al.*, 1996). Under these conditions, the replication reaction in the nuclei was almost completely inhibited, as assessed by the apparent absence of incorporation of dCTP into the chromatin. In contrast, both the XMCM3 and XMCM5 signals decreased from the nuclei regardless of the presence of the inhibitor (Figures 4B and 5), although the rate of disappearance was slower than that obtained in the absence of aphidicolin (Figure 4A). These results suggest the functional difference between XMCM3–5 and other XMCM proteins having Zn finger-like motifs on the initiation of replication.

Physical and functional association of XMCM proteins

The similarity in the behavior of XMCM proteins during the cell cycle suggested their physical association in the egg extracts. This is evidently supported by the co-precipitation of six XMCM proteins with an anti-XMCM3 antibody (Figure 1A). Figure 6 further demonstrates the physical and stoichiometric association of XMCM proteins in the egg extract. Upon immunodepleting XMCM3 from the extract with anti-XMCM3 antibody, other members of the XMCM family are co-depleted from the extracts. The quantification of immunoblots of each XMCM proteins indicated that >90% of XMCM proteins were depleted from the extract except for XMCM2 (75% depletion) and XMCM6 (87% depletion) (Figure 6A). Evidence for the physical association of XMCM proteins in the egg extracts was also supported by immunoprecipitation of the egg proteins by a specific antibody against each of the XMCM proteins. Figure 6B shows that the immunoprecipitates obtained by anti-XMCM2, -XMCM5 and -XMCM7 antibody showed similar protein compositions to each other. Essentially the same results were obtained with affinity purified anti-XMCM4 and anti-XMCM6 antibody (see Figure 6C). The immunoblotting of the precipitates demonstrated that they contained all six XMCM proteins. We further examined the stoichiometric ratio of proteins associated with anti-XMCM3 or anti-XMCM4 immunoprecipitates by densitometric measurements of stained protein bands. An apparent molecular ratio of p112, p100, p92 and p90 was roughly calculated as 1.0:3.1:1.2:0.9. All these results indicate that six XMCM proteins form a physical complex in the egg extract.

A physical complex of six XMCM proteins was dissociated into two fractions by stepwise elution of the immunoprecipitates (Figure 1B). With the anti-XMCM3 antibody, we could separate an XMCM3–5 complex from the other MCM proteins with Zn finger-like motifs. Figure 6C shows a tight association between some XMCM proteins containing a Zn finger-like motif. The high salt eluates of immunoprecipitates with anti-XMCM4 or XMCM6 antibody contained p112 (XMCM2), p100 and p92 (XMCM5) proteins. Proteins remaining on the precipitates were p100 and p90 (XMCM7). Immunoblotting of these fractions with anti-XMCM3, -XMCM4 and -XMCM6 antibody revealed that the major constituent of p100 in high salt eluates of these immunoprecipitates was XMCM3. XMCM4 and XMCM6, together with XMCM7, remained in the precipitates washed with high salt. In contrast, most of XMCM2, XMCM4, XMCM6 and XMCM7 were eluted in high salt from anti-XMCM3 immunoprecipitates. These results showed that two tightly associated protein complexes were formed *in vitro*, one consisting of XMCM3 and XMCM5 and the other consisting of XMCM4, XMCM6 and XMCM7.

Figure 7A shows that the dissociated sub-fractions of the XMCM protein complex were not functionally active in supporting replication. The replication activity of S-phase egg extract was abolished by anti-XMCM3 antibody immunoprecipitation, which resulted in the almost com-

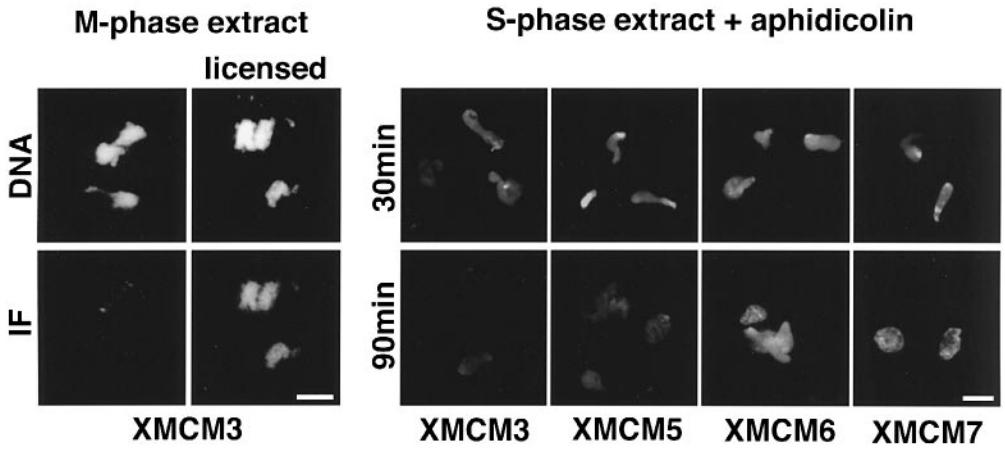

Fig. 5. Persistent association of XMCM proteins with chromatin in the absence of replication activity in the egg extract. M-phase extract: sperm chromatin with (licensed) and without (unidentified) incubation in the membrane-depleted S-phase extract for 20 min were incubated in the M-phase egg extract for 60 min at 23°C. Samples were then fixed and processed for the fluorescent detection of DNA and XMCM3 protein. S-phase extract: sperm chromatin was incubated in the S-phase extract in the presence of 20 μg/ml aphidicolin for 30 and 90 min at 23°C, and each sample was fixed and processed for the fluorescent detection of XMCM proteins. Scale bar 20 μm.

plete depletion of XMCM3 and other XMCM proteins. Reconstitution with the high salt eluate of the anti-XMCM3 immunoprecipitate, whose major constituent was XMCM proteins containing a Zn finger-like motif, or with an alkali eluate of the XMCM3–5 complex, did not rescue the activity, but rather inhibited the residual replication activity (Figure 7A columns 1–3). Mixing of equal quantities of both fractions reconstituted the replication activity (Figure 7A column 4). Under these conditions, the ratio of each XMCM protein became near unity. These results suggest that the replication activity requires stoichiometric amounts of the six XMCM proteins in the egg extract.

Permeability of XMCM3 protein to the nuclear envelope

We have previously shown that recombinant as well as endogenous XMCM3 protein was not transported into the nuclei in the egg extract (Kubota *et al.*, 1995). These experiments did not exclude the possible entry of XMCM3 proteins into the nuclei. Therefore, we prepared an XMCM3-depleted S-phase extract, which was capable of forming nuclei upon the addition of sperm chromatin. This allowed us to test whether or not the XMCM protein complex could cross the nuclear envelope to rescue the replication activity of the depleted extract. First, we measured the replication activity of XMCM3-depleted extract reconstituted with the XMCM protein complex added before and after nuclear formation. As shown in Figure 7B column 2, the replication activity was rescued when the protein complex either in the form of affinity purified protein (shaded column) or in membrane depleted S-phase extract (open column) was added before nuclear formation. After nuclear formation, we obtained essentially the same activity as with the depleted extract alone (compare columns 1 and 3). These results indicate that the XMCM protein complex cannot rescue replication activity after nuclear formation is complete.

Next we examined whether the nuclear formation is essential for preventing the association of XMCM proteins with chromatin. For this purpose, we prepared two types of XMCM3-depleted extract, one from whole egg extract, and the other from membrane- depleted extract. With XMCM3-depleted whole egg extract, neither the nuclear signal of XMCM3 nor the incorporation of bio-dUTP was detected 90 min after the addition of sperm chromatin, though distinct nuclear structures were detected under phase contrast microscopy (data not shown). When the extract was reconstituted with the XMCM protein complex (membrane-depleted S-phase extract) before the addition of sperm chromatin, a prominent XMCM3 signal was detected in the nuclei formed 45 min after addition of sperm chromatin to the reconstituted extract (Figure 8), and further incubation resulted in the incorporation of bio-dUTP concomitant with the decrease in XMCM3 signal in the nuclei (data not shown). When the XMCM protein complex was added after the formation of nuclei in the XMCM3-depleted extract, we detected a very faint signal of XMCM3 in the nuclei upon further incubation in the extract (Figure 8), and little incorporation of bio-dUTP into the nuclei was observed upon incubation in the reconstituted extract for 60 min (data not shown). In contrast, XMCM proteins could associate with chromatin that had previously been incubated in XMCM3-depleted extract in the absence of nuclear formation. The membrane-depleted extract was not capable of supporting nuclear formation, but was able to license sperm chromatin added to the extract (Kubota and Takisawa, 1993). Figure 8 shows that association of XMCM3 with chromatin was equally induced when the depleted extract was reconstituted with the XMCM protein complex before and after incubating sperm chromatin in the XMCM3-depleted extract. These results indicate that nuclear formation prevented the assembly of XMCM protein complex with chromatin.

Discussion

The MCM/P1 family of proteins has been implicated in the regulation of initiation of replication in eukaryotes (reviewed in Tye, 1994; Chong *et al.*, 1996; Kearsey *et al.*, 1996). Increasing evidence further suggests that it is involved in a mechanism which restricts the replication of DNA to exactly once in each cell cycle (reviewed in Chong *et al.*, 1996; Kearsey *et al.*, 1996; Romanowski

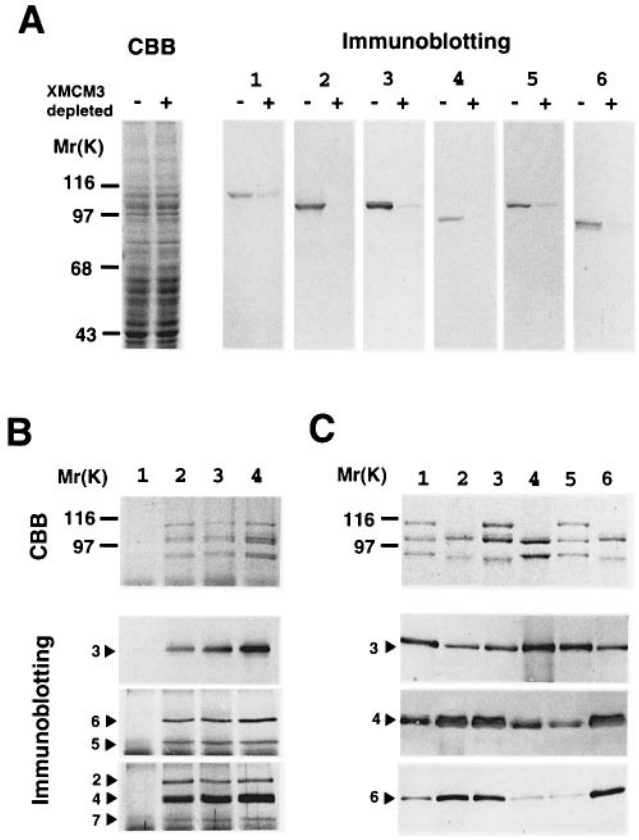

Fig. 6. Physical association of six XMCM proteins. (**A**) Co-depletion of six XMCM proteins from the egg extract with anti-XMCM3 antibody. The membrane depleted S-phase extract was incubated with control (–) or anti-XMCM3 (+) antibody-conjugated protein A beads, then resolved by SDS–PAGE. Proteins were visualized by Coomassie blue staining and XMCM proteins in each extracts were visualized by immunoblotting with affinity purified antibodies against XMCM2 (1), XMCM3 (2), XMCM4 (3), XMCM5 (4), XMCM6 (5) and XMCM7 (6). (**B**) Immunoprecipitation of six XMCM proteins from the egg extract using specific antibodies against various XMCM proteins. The membrane-depleted extract was incubated with control (1), anti-XMCM2 (2), anti-XMCM5 (3) and anti-XMCM7 (4) antisera, and the proteins bound to the antibodies were recovered with protein A beads. The proteins bound to the beads were resolved with SDS–PAGE, and the protein composition was visualized by Coomassie blue staining. Each protein was identified by affinity purified antibodies against each XMCM protein (indicated by numbers of the MCM/P1 family with arrowheads). (**C**) Dissociation of XMCM2 protein from XMCM immunoprecipitates. The membrane-depleted S-phase extract was incubated with anti-XMCM4 (1 and 2), anti-XMCM3 (3 and 4), and anti-XMCM6 (5 and 6) antibody and the proteins bound to the antibodies were recovered with protein A beads. The beads were then incubated with high salt buffer containing 1.0 M NaCl. The proteins eluted with the high salt buffer (1, 3 and 5) and those remaining on the beads (2, 4 and 6) were resolved by SDS–PAGE, and visualized by Coomassie blue staining. The identity of proteins migrating at ~100-kDa was investigated by immunoblotting with affinity purified anti-XMCM3, anti-XMCM4, and anti-XMCM6 antibodies (indicated by numbers of the MCM/P1 family with arrowheads).

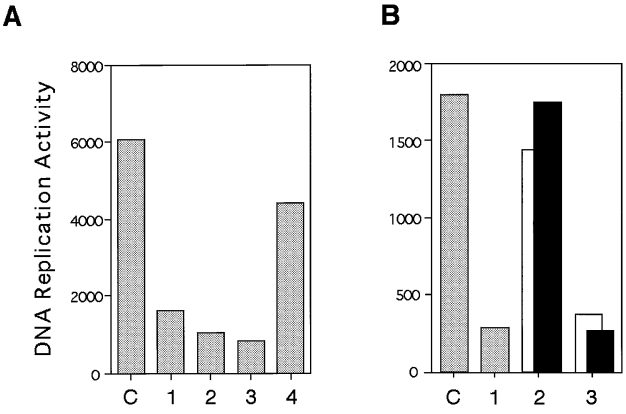

Fig. 7. Requirement of a complex of six XMCM proteins for the replication activity of the S-phase egg extract. (**A**) Replication activity of the XMCM3-depleted extract reconstituted with and without XMCM proteins. The S-phase egg extract was mock depleted (column c), or depleted with anti-XMCM3 antibody-conjugated protein A beads (columns 1, 2, 3 and 4). The depleted extract was reconstituted with 1/5 volume of dialysis buffer alone (columns c and 1), the high salt eluate of the XMCM3 associated proteins shown in Figure 1A, lane 1 (column 2, 96 ng/μl depleted extract), the alkaline eluate of the XMCM3 associated proteins shown in Figure 1A, lane 2 (column 3, 88 ng/μl depleted extract), or a combination of the high salt and the alkali eluates (column 4, 48 ng high salt eluate; 44 ng of alkali eluate per μl of depleted extract). The replication activity of each extract was determined as the incorporation of [α-^{32}P]dCTP into sperm DNA during a 90 min incubation in the extract, and the average of duplicated experiments is shown. Estimated amounts of DNA synthesized in mock-depleted extract was ~60% of input DNA. Essentially similar results were obtained in two independent experiments. (**B**) Requirement for a six XMCM protein complex for replication before nuclear formation. XMCM3 depleted extract, incubated with sperm chromatin for 0 (column 2) or 45 min (column 3), was reconstituted with a complex of six XMCM proteins (160 ng/μl depleted extract) (shaded column) or membrane depleted S-phase extract (0.2 μl/μl depleted extract) (open column), and replication activity of the reconstituted extracts (column 2 and 3) was measured 90 min later. Replication activity of mock- (column c) and XMCM-depleted (column 1) extracts was measured 90 min after addition of sperm chromatin.

and Madine, 1996). Previous experiments with the *Xenopus* cell-free system showed that *Xenopus* MCM3 is one of the essential components required for the licensing activity of the egg extracts (Chong *et al.*, 1995; Kubota *et al.*, 1995; Madine *et al.*, 1995a,b). Here, we addressed three critical questions about the role of XMCM3 in licensing replication. The first question concerned the identity of proteins associated with XMCM3, which apparently form a physical complex in the egg extract. The second question concerned the function of the proteins associated with XMCM3 in replication. The third question concerned the contradictory features of the permeability of XMCM3 proteins to the nuclear envelope. Employing specific antibodies against each of the proteins associated with XMCM3, we have identified five immunologically distinct proteins as members of the MCM/P1 family. Further studies on the behavior of XMCM proteins during the cell cycle, the physical interaction of XMCM proteins in the egg extract, and the reconstitution of replication activity with an XMCM protein complex revealed that a physical complex of six XMCM proteins plays a central role in the licensing of replication observed in the egg extract. The conserved structure and function of MCM/P1 proteins further suggest that the cell cycle regulated re-association of MCM/P1 protein complex with chromatin is a key feature of the replication licensing phenomena in the eukaryotic cell cycle.

Six members of the Xenopus MCM/P1 family of proteins in the egg extract

Previous studies suggest the presence of six members of the MCM/P1 family of proteins in eukaryotic cells (reviewed in Chong *et al.*, 1996; Kearsey *et al.*, 1996).

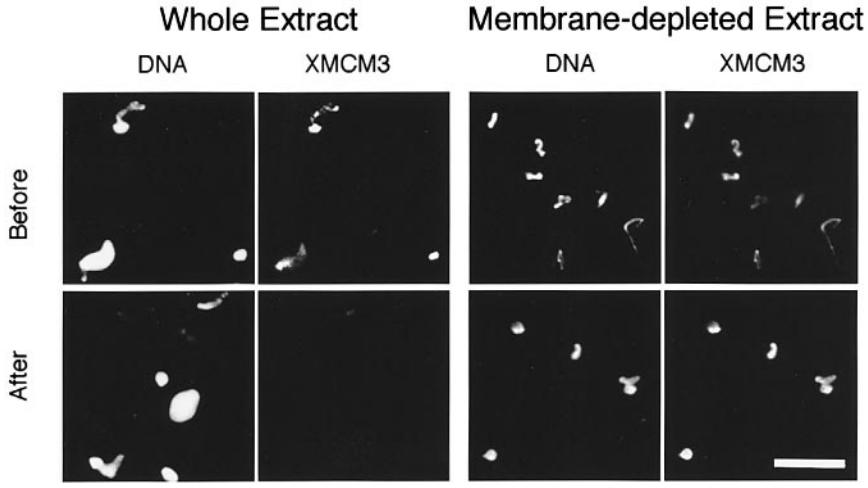

Fig. 8. Nuclear formation prevents the accumulation of XMCM3 into the nuclei. The XMCM3-depleted extracts prepared from whole egg extract or membrane-depleted extract were incubated with sperm chromatin for 0 (before) or 35 min (after), and the extract was reconstituted with four volume of the membrane-depleted S-phase extract and localization of XMCM3 protein was examined 45 min after addition of sperm chromatin. Nuclear DNA and XMCM3 were visualized by Hoechst, and rabbit anti-XMCM3 antibody followed by FITC labeled anti-rabbit IgG, respectively. Scale bar 50 μm.

The complete sequence of the genome of the budding yeast, *S. cerevisiae*, has provided us with evidence for the universal conservation of six MCM/P1 proteins in eukaryotes. We have searched for the yeast homologs of the six XMCM proteins with BLASTX or TBLASTN over the complete genome sequence, and found that each of the predicted amino acid sequences of *Xenopus* proteins showed the highest homology to six yeast gene products, five of which have been identified as MCM/P1 proteins and one of which is coded by an unidentified gene (feature name YGL201C) showing the highest similarity to fission yeast mis5 (SpMCM6). By examining the degree of amino acid homology between each XMCM proteins and the yeast gene products, we have found that each of the XMCM proteins showed the highest homology to a single different yeast gene product. Essentially the same family members were assigned to the previously reported sequences of MCM/P1 proteins of different organisms (see Table I). In addition, we could find no other protein sequences showing significant similarity to XMCM proteins in the yeast databases. These results reinforced the conclusion of the universal conservation of six members of the MCM/P1 protein family in eukaryotes.

The identification of the primary structures of six XMCM proteins revealed several unique sequence motifs in the MCM/P1 proteins, in addition to previously reported motifs such as a putative NTPase motif in domain 3, and a Zn finger-like motif between domains 1 and 2 (reviewed in Tye, 1994; Kearsey *et al.*, 1996). One unique sequence found in this work is a heptad K/R repeat in conserved domain 5. A very similar repeat was found in the three putative ORFs of the *Methanococcus* genome (MJ0363, MJ0961, and MJ1489), showing striking homology to MCM/P1 proteins of eukaryotes (Bult *et al*., 1996). These amino acids in an α-helical structure will form aligned positively charged groups. In addition, most of these basic amino acids were surrounded with acidic amino acids, thus indicating the presence of highly oriented charged residues in domain 5. The exact function of this repeat is not known, but one possible function may be for interaction with other proteins, including other members of the MCM/P1 proteins. Interestingly, Kimura and co-workers showed that domain 5 is apparently required for the interaction between mammalian MCM3 and MCM5 (H.Kimura, personal communication). Further studies on the function of each conserved domain will enable us to clarify the function of MCM/P1 proteins in replication.

Physical complex of six XMCM proteins in the egg extract

Recent studies on mammalian and *Drosophila* MCM/P1 proteins indicate the presence of various forms of physical complex of MCM/P1 proteins (Burkhart *et al.*, 1995; Kimura *et al.*, 1995; Musahl *et al.*, 1995; Su *et al.*, 1996). Here we show that the six XMCM proteins form a physical complex in the egg extract. The complex of six XMCM proteins could be dissociated into at least two components *in vitro*, and our data showed that each dissociated component could not rescue the replication activity of the XMCM3-depleted extract. Neither a stable complex of XMCM3 and XMCM5 protein nor the other complex of XMCM proteins containing Zn finger-like motifs (XMCM2–4–6–7) could rescue the replication activity of the depleted extract. In contrast, the combination of these two fractions could reconstitute the replication activity of the depleted extract, suggesting that the functions of the XMCM proteins are not redundant. These results are consistent with the genetic studies on the yeast MCM/P1 family of proteins, showing that each of the MCM genes is essential for viability (reviewed in Tye, 1994; Chong *et al.*, 1996; Kearsey *et al.*, 1996).

The distinct function of the XMCM proteins was indicated by the formation of different complexes *in vitro*. A tight association of MCM3 and MCM5 proteins has previously been shown in mammalian and yeast cells (Burkhart *et al.*, 1995; Kimura *et al.*, 1995; Lei *et al.*, 1996), and similar result was obtained with the dissociated component of purified RLF-M (Thömmes *et al.*, 1997). The association of MCM2–4–6–7 proteins has been reported with mammalian cells (Musahl *et al.*, 1995) and

in the *Drosophila* embryo (Su *et al.*, 1996). In addition, we found that XMCM2 was not tightly associated with XMCM4–6–7, since XMCM2 protein was dissociated from the other MCM proteins under the high salt conditions. A weak interaction between the MCM2 protein and other MCM proteins has been observed in mammalian (Musahl *et al.*, 1995) and yeast cells (Lei *et al.*, 1996). All these results indicate that the mode of physical interaction between MCM proteins as well as their function appears to be conserved throughout phyla.

The tight physical association of XMCM3 and 5 conspicuously resembles the behavior of these proteins during the cell cycle. Both XMCM3 and XMCM5 proteins behaved differently from XMCM2–4–6–7 proteins. The immunofluorescence signal of XMCM5 as well as that of XMCM3 in the nuclei decreased rapidly upon the formation of nuclei, and this decrease was rather insensitive to the presence of aphidicolin. In contrast, the signal of XMCM2–4–6–7 proteins remained in the nuclei in the presence of aphidicolin. Since XMCM3–5 proteins as well as XMCM2–4–6–7 proteins apparently remained on the chromatin in the presence of aphidicolin, the decrease in the signal of XMCM3–5 proteins in the presence of the polymerase inhibitor presumably reflects their dissociation from XMCM2–4–6–7 proteins with a conformational change in the epitope site of these proteins during the reaction, independently of the chain elongation stage of replication. Previous reports with mutations of SpMCM5 (*nda4*) suggest that such conformational changes may occur early in S-phase, possibly before the initiation of replication (Miyake *et al.*, 1993). A shift to the non-permissive temperature in *nda4* mutants but not in *nda1* (MCM2) mutant cells revealed a reversible phenotype arrested at G_1/S phase. One simple explanation for this is that the *nda4* mutation led to a defect in the process before the start of an irreversible replication reaction and possibly at the formation of the initiation complex. Recent reports on the genetic interaction between the origin binding protein, ORC and the MCM/P1 proteins (Li and Herskowitz, 1993; Loo *et al.*, 1995) further suggests a crucial role for MCM/P1 proteins in the formation of the initiation complex.

Permeability of the nuclear envelope to XMCM3 proteins

The present results support our previous findings that the entry of XMCM3 protein into the nuclei was prevented by the nuclear envelope, thus ensuring that re-replication is prevented during a single cell cycle. However, these results obviously contradict previous results obtained by Madine *et al.* (1995a,b). According to their reports, XMCM3 in the egg extract could enter nuclei assembled in XMCM3-depleted extract, and thus support replication after the formation of nuclei (Madine *et al.*, 1995a). We occasionally observed that the intact nuclear envelope was not formed in the XMCM3-depleted extract by incubating the sperm chromatin for 45 min. This is possibly due to dilution of the extract, and further incubation was required to form an intact nuclear envelope. With such extract, we could reconstitute the replication activity by adding XMCM protein complex 45 min after incubating the sperm chromatin in the depleted extract (data not shown). This result together with that obtained with the membrane-depleted extract (Figure 8) exclude the possible involvement of XMCM proteins during remodeling of the sperm chromatin. But the contradictions between the rescue experiments of Madine *et al.* (1995a) and ours could not be explained, since Madine *et al.* (1995a,b) reported that the nuclear envelope is intact during the course of their experiments. Phenomenologically, all these results could be explained by regulated entry, but not by free entry of XMCM proteins into the nuclei. It should also be stressed that the assumption of the non-permeable nature of any other factor involved in licensing replication does not explain the following results. (i) The permeabilization of the nuclear envelope was required for initiating replication of G_0 nuclei in the egg extract (Leno and Munshi, 1994), while the replication of G_0 nuclei normally occurs without the breakdown of the nuclear envelope upon growth stimulation of G_0 cells. (ii) Nuclear DNA can be re-replicated without an apparent M-phase under certain conditions which disturb the regulation of cell cycle progression (reviewed in Coverley and Laskey, 1994; recent reports by Nishitani and Nurse, 1995; Heichman and Roberts, 1996). (iii) In most lower eukaryotes, the nuclear structures apparently remain intact during the cell division cycle (Heath, 1980). These and other results led us to consider that the permeability of the nuclear envelope to a specific factor may play a crucial role only in the embryonic cell cycle such as in *Xenopus* eggs, but not in the somatic cell cycle, in which XMCM3 and its homologs are transported into the nuclei. In *Xenopus* embryos, an excess of protein factors involved in the initiation of replication are present in cytoplasm, so that the free entry of these factors should be strictly prohibited to prevent the re-replication of DNA.

Role of XMCM protein complex in the licensing of replication

The conserved feature of the licensing phenomena in mitotic cell cycle is the regulated association of MCM/P1 proteins with the chromatin. MCM/P1 proteins became associated with chromatin on exit from M-phase and dissociate from it upon the progression of the replication. These features suggest that MCM/P1 proteins are involved in a mechanisms distinguishing replicated from unreplicated chromatin during the cell division cycle, and thus in the licensing of replication. Then, the inappropriate re-replication of DNA could be explained by either defects in the dissociation of MCM/P1 proteins from chromatin, or the untimely association of MCM/P1 proteins with chromatin. At present, it is not known whether the inactivation of MCM proteins is directly correlated with their dissociation from chromatin. In any case, inactivation of the licensing activity in S- and G_2 phase of nuclei is inevitable for cells, whose MCM/P1 proteins remain in the nucleus. To inactivate the licensing activity with appropriate timing, it should be regulated by cell cycle control machinery. The multitude of target sites in MCM/P1 proteins for various kinases, and cell cycle regulated phosphorylation and dephosphorylation of some members of the MCM/P1 family (Kimura *et al.*, 1994; Todorov *et al.*, 1995; Coué *et al.*, 1996) further suggest that the regulation of the phosphorylation state of MCM/P1 proteins is one of the key events regulating their activity.

Future investigation will enable us to clarify the exact role of MCM/P1 proteins in the licensing of replication.

Materials and methods

Immunoaffinity purification of XMCM3 associated proteins

The membrane-depleted S-phase extract of *Xenopus* eggs was incubated with an equal volume of protein A beads conjugated with anti-XMCM3 antibody for 30 min at 4°C. The beads were sedimented by a brief centrifugation at 10 000 *g* for 15 s, washed with EB (100 mM KCl, 2.5 mM $MgCl_2$ and 50 mM HEPES–KOH pH 7.5) containing 0.1 M NaCl, then the proteins bound to the beads were eluted with EB containing 0.8 M NaCl (high salt eluate), followed by 1.0 M NaCl containing 50% ethylene glycol, and 50 mM CAPS at pH 11.0 (alkali eluate). Each fraction was concentrated, and dialyzed against EB containing 10% glycerol and stored at –20°C.

Production and purification of antibodies

Antibodies against proteins associated with XMCM3 were prepared as follows. Protein fractions (high salt and alkali eluates) were separated by SDS–PAGE, and protein bands corresponding to p112, p102, p92 and p90 were excised from the gel. Female mice were immunized with these proteins by the standard protocols. Generation of antibodies was checked by immunoblotting egg extract and immunoprecipitates, and the mice producing the antibodies were repeatedly boosted for 4–5 week intervals. The antiserum obtained after the final boost was generally diluted 10 000-fold for immunoblotting. Each antiserum was further purified with GST-XMCM proteins immobilized on CNBr-activated sepharose-4B (Pharmacia), according to the protocols supplied by the manufacturer. The affinity purified antibodies were dialyzed against 0.1 M NaCl, 20 mM HEPES pH 7.5 containing 0.02% NaN_3, concentrated and stored at 4°C.

Immunoprecipitation and immunoblotting

Egg proteins were immunoprecipitated by the antibodies against various XMCM proteins as follows. To prepare samples for immunoblotting and protein detection, 100 µl of the membrane-depleted extract was incubated with 20 µl of antisera against various XMCM proteins for 30 min at 0°C. The proteins bound to the antibodies were recovered by incubating the extract with 20 µl of Affi-Prep Protein A Matrix beads (Bio-Rad) for 30 min at 0°C. The beads were collected by a brief centrifugation at 10 000 *g* for 15 s, then washed with EB containing 0.1 M NaCl. Total proteins bound to the beads were solubilized in SDS–PAGE sample buffer. The high salt eluate was prepared by adding 200 µl of EB containing 1.0 M NaCl to the washed beads, then separating them from the buffer by centrifugation at 10 000 *g* for 5 min. The supernatant was used as the high salt eluate. Proteins remaining in the beads were solubilized with the sample buffer. Immunoblotting of each sample proceeded as described (Kubota and Takisawa, 1993). Quantification of the blots was performed by a laser scanner (Sharp, JX-325M) with NIH Image software.

Cloning and sequencing of cDNA

A lambda ZAP-derived cDNA library of *Xenopus* oocytes mRNAs was screened using anti-*Xenopus* p112, p102, p92, p90 antisera, or mouse cdc21 cDNA as probes. From 5×10^5 plaques, we isolated one clone for p112, p102 and the mouse cdc21 homolog, five for p92, and six for p90. Using the cDNAs as probes, we further screened the library to obtain several clones for each corresponding protein. Restriction enzyme mapping and the partial sequencing of each cDNA clone revealed that most positive clones identified by each probe were derived from the same mRNA species. One of six clones of p90 had several unique restriction sites. Each clone containing the largest cDNA for p112, p102, p92, the mouse cdc21 homolog, and two different clones for p90 were sequenced for both strands by the automatic DNA sequencer (ABI, 373A).

Bacterial expression of Xenopus MCM proteins

GST–*Xenopus* MCM proteins were prepared as described. To prepare full-length recombinant XMCM2, 5, and 6, the initiating codon in each cDNA clone was converted into various restriction sites by PCR, where the 5′ primers were 5′-CGTGGATCCGGCGCGCCCATGGCGGATT-CTTCAGAGTCATTTA, 5′-CGTGGAATTCGAGGCGCGCCCATGT-CGGGATTTGATGATCTTGGA, 5′-CGTGGATCCATGGAATTGGG-AGGCCCTG and the 3′ primers were 5′-CATCAAATATCTTAA-GCATTTCAGCA, 5′-ATCAAAGCTAGCCAGGTCCTCCA, 5′-GAGC-TCGCGAATCTTTTG, for XMCM2, XMCM5 and XMCM6, respectively. The PCR products were subcloned into pBluescript and sequenced. The appropriate restriction enzyme fragments (*Bam*HI–*Afl*II, *Eco*RI–*Nhe*I and *Bam*HI–*Nru*I for XMCM2, XMCM5 and XMCM6 fragments, respectively) were then ligated into pBluescript carrying corresponding XMCM cDNAs digested with the appropriate restriction enzymes. The full length cDNAs of XMCM2, 5 and 6 without a stop codon, the *Eco*RI–*Xho*I digested fragment of XMCM7 and the *Eco*RI–*Xho*I and *Xho*I–*Xho*I fragments of XMCM4 were ligated into pGEX vectors, which were then transfected with *Escherichia coli* strain PR745. The transformed cells were grown at 20°C and harvested, and lysed in a French press. GST fusion proteins were purified using glutathione–Sepharose 4B (Pharmacia LKB Biotechnology). The purity of the recombinant proteins was assessed by SDS–PAGE and visualized by Coomassie blue staining. Most of the recombinant proteins were over 80% pure.

Reconstitution of XMCM3-depleted egg extract with affinity purified XMCM proteins

The S-phase egg extract was incubated with a 1/10 volume of protein A beads conjugated with affinity purified anti-XMCM3 antibody for 30 min at 0°C. Following the removal of the protein A beads, the extract was repeatedly depleted with the antibody-conjugated protein A beads. After removing the beads, the extract was reconstituted with 1/5 volume of affinity purified XMCM proteins, or four volumes of the membrane-depleted extract. The formation of nuclei in the extract was monitored by observing them under phase contrast microscopy. The replication activity of the egg extract was measured as the incorporation of [α-^{32}P]dCTP into DNA, and approximate DNA synthesis in ng/µl of each samples were estimated by multiplying the percentage total counts incorporated into DNA by a factor of 0.654 (Blow and Laskey, 1986).

Immunofluorescence microscopy

Samples for immunofluorescence microscopy was prepared as described (Kubota and Takisawa, 1993). Immunofluorescence of each XMCM proteins in nucleus was quantified by comparing fluorescence intensities collected at identical settings on cooled CCD camera (Photometrics, PXL). In order to obtain average image, we selected a frame containing 20–30 nuclei, and the fluorescence intensities of all nuclei were measured to calculate average fluorescence intensity per nucleus. Fluorescence images were collected as 4096 gray scales with IPLab Spectrum (Signal Analytics Corporation), and these digital images were converted into TIFF images with 256 gray scales. The fluorescence images of Hoechst staining were taken to determine the area of each nucleus. The average intensity per pixel of each nucleus was subtracted with that of background level, and total pixel intensities of each XMCM fluorescence signals per nucleus were estimated with NIH Image software.

Miscellaneous methods

Xenopus egg extract, membrane-depleted extract, and demembranated sperm chromatin were prepared as described (Kubota and Takisawa, 1993).

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