In Vivo Interaction of Human MCM Heterohexameric Complexes with Chromatin

POSSIBLE INVOLVEMENT OF ATP*

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The MCM protein family, which consists of at least six members, has been implicated in the regulatory machinery causing DNA to replicate once in the S phase. Mammalian MCM proteins are present in the nucleus in two different forms, one extractable by nonionic detergents and the other resistant to such extraction. The latter is assumed to be tightly associated with nuclear structures and released at the time of initiation of replication. However, details of the mode of binding remain unclear. In the present study, we found that, in nonionic detergent-permeabilized nuclei, the association of human MCM (hMCM) proteins with them could be stabilized by the addition of ATP. The hMCMs bound to the nuclei in the presence of ATP were released by digestion with nucleases, suggesting that they are chromatin-associated. The nuclease-directed solubilization of the chromatin-bound hMCMs thus provided a means to analyze them as well as soluble hMCMs by co-immunoprecipitation. The results indicate that the six hMCM members exist as heterocomplexes, whether bound or unbound. We therefore propose that hMCM proteins may function in DNA replication as heterohexamers associated with chromatin and that ATP is possibly involved in the association. Nuclease digestion-immunoprecipitation techniques of the type described here should facilitate further elucidation of the mode of interaction between hMCMs and chromatin.

Identification of DNA sequences able to act as origins of replication (autonomously replicating sequences; ARS) has allowed extensive analysis of the initiation step in the budding yeast. The MCM protein family, which consists of at least six members, was originally identified because of its involvement in the initiation of DNA replication at ARS (1–8) and later found to be conserved through eukaryotes (9–18). Although definite functions of the MCM proteins remain largely unknown, they have been implicated in the regulatory machinery allowing DNA to replicate only once during S phase (reviewed in Refs. 19 and 20).

In mammalian cells, MCM proteins are present in the nuclei in two different forms that can be differentiated by nonionic detergent extraction, one soluble and extractable and the other tightly associated with the nucleus, which is resistant to such extraction. The level of mammalian MCMs does not vary greatly during the cell cycle, but the nucleus-bound form gradually becomes dissociated from nuclear structures with progression through the S phase (21–25). It is now assumed that it is associated with prereplicative chromatin and released at the time of initiation of replication; the soluble form existing abundantly in G2 nuclei is considered inactive and no longer capable of binding to chromatin. Based on the finding that the nucleus-bound MCMs are liberated by nuclease treatment, they are thought to be linked to chromatin structures, but some controversy still remains in this regard (21, 22, 26). It has also been suggested that replication factories are connected with the nuclear matrix, which is not released by nuclease digestion (reviewed in Ref. 27; Ref. 28).

Whereas the MCM proteins share substantial homology, in budding yeast, each type functions in an ARS-specific manner to play indispensable roles in chromosomal replication (3–8). Similarly, in human cells the function of the hCDC47 form does not appear to be compensated for by other members (29). On the other hand, yeast MCMs exhibit genetic interactions (4, 7) and, just recently, have been shown to physically associate with one another (30). In Xenopus or Drosophila egg extracts, all of the MCM members appear to exist as heterocomplexes (15–17, 31, 32). In mammalian cells, interactions among MCMs have been studied mainly using nonionic detergent-extractable fractions, where CDC46 and MCM3 assemble into a complex (21, 23, 26), and CDC47, cdc21, and Mis5 form another complex, which relatively weakly associates with MCM2 (33, 34). Although such studies have provided useful information for understanding mammalian MCMs, it is very important that we analyze their nucleus-bound counterparts, whose biological and biochemical properties are presumably distinct. Indeed, the state of phosphorylation is known to be different between the two types, seemingly affecting nuclear association-dissociation (21, 22) and perhaps also influencing protein-protein interactions. In the present study, we therefore investigated the properties of the nucleus-bound form of human MCM (hMCM) proteins.

MATERIALS AND METHODS

Cells, Buffers, and Reagents—HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum.

The buffer used for preparation of cell lysates and the immunoprecipitation procedure was modified CSK buffer (35) (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) containing 0.1 or 0.5% Triton X-100 (0.1%TX-100mCSK or 0.5%TX-100mCSK, respectively). ATP, GTP, CTP, and UTP were purchased from Sigma; ATPγS and AMP-PNP were from Calbiochem.

The preparation, purification, and specificity of rabbit anti-hCDC47 (human CDC47) antibodies were described previously (24).

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The abbreviations used are: ARS, autonomously replicating sequences; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen; PIPES, 1,4-piperazinediethanesulfonic acid; ATPγS, adenosine 5’-O-(thiotriphosphate); AMP-PNP, adenosine 5’-(β,γ imido)triphosphate.

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murine MCM3 (mMCM3) antiserum or antibodies affinity-purified with full-length mMCM3, anti-mcde21 antisera, and anti-mCD46 antisera were kindly provided by Dr. H. Kimura (Hokkaido University, Sapporo, Japan). Rabbit anti-Xenopus MCM2 (XMCM2) antisera was kindly provided by Dr. S. Miyake (Toho University, Tokyo, Japan). All of these antibodies recognized not only cell cycle-regulated cell nuclear antigen (PCNA) mouse monoclonal antibody at 20 μg/ml (PC10, Boehringer Mannheim). The blots were then probed with peroxidase-labeled goat anti-rabbit IgG or anti-mouse IgG antibodies (Zymed) and visualized using the ECL system (Amersham Corp.). The intensity of signals visualized on films was measured with a CS-930 chromatoscanner (Shimadzu, Japan). Results

Stabilization of the Nuclear Association of hMCM Proteins by ATP—During the course of the present analyses, we found that the nucleus-bound hMCM proteins were released from the extracted nuclei by heating. Immunoblotting analysis of the levels of hCDC47 protein in the nuclear pellets and the soluble supernatants separated after incubating Triton X-100-extracted HeLa cell nuclei revealed that, by incubation at 25 °C for 40 min, about half of the nucleus-bound hCDC47 was liberated into the supernatant, and at 33 °C for 40 min, almost all was liberated (Fig. 1A). With parallel immunoblotting using anti-mMCM3 antibodies, hMCM3 was also found to be dissociated with similar kinetics (Fig. 1C). PCNA, the auxiliary protein of DNA polymerase δ (37), is also known to have a detergent extraction-resistant nucleus-bound form (38), the levels of which are cell cycle-regulated during S phase but differently from hMCM proteins (24). As shown in Fig. 1A, the nucleus-bound PCNA was also found to be dissociated from the nuclei in a temperature-dependent manner. For all three proteins, neither detectable proteolytic products nor remarkable reduction in the total amount were found after heating.

There are several possible reasons for the observed thermostability of the nuclear association of these proteins. Interestingly, for hMCM proteins, the addition of ATP to the reaction mixture inhibited their dissociation. In contrast to the control case, in the presence of 0.1 mM ATP, almost all hCDC47 protein was retained in the nuclear pellet even after incubation at 33 °C for 40 min (Fig. 1B). ATP-dependent retention was also observed for hMCM3 (Fig. 1C) and hcde21 (data not shown).

In contrast, the addition of ATP failed to inhibit the thermostability of the PCNA nuclear association (Fig. 1B), indicating that the effect of ATP is not nonspecific. In all of the cases described above, the core histones were detected only in the nuclear pellet fractions, irrespective of the incubation temperature or the presence of ATP (Fig. 1B).

To examine the specificity of the ATP requirement for the nuclear association of hMCMs, the experiments were repeated in the presence of three other ribonucleoside triphosphates. As shown in Fig. 2A, none of them could maintain the association of hCDC47 as effectively as ATP. Similar results were also obtained for hMCM3 (data not shown). To address the role of ATP, two ATP analogs were tested for their ability to function in place of ATP. At comparable concentrations, both ATPγS and AMP-PNP, which has a nonhydrolyzable βγ bond, could maintain the association of hCDC47 (Fig. 2B) or hMCM3 (data not shown). This indicates that the hydrolysis of ATP βγ bonds, required for protein phosphorylation or ATPase reaction.
The incubation time for the nuclei was 40 min.

for detection of core histones. Also subjected to 15% SDS-PAGE followed by Coomassie Blue staining

100 mCSK buffer, which contains 1 mM MgCl₂, and before in-

X-100-extracted nuclei were prepared as usual with 0.5% TX-

undesired nuclear lysis by removal of divalent cations, Triton

ions, especially Mg²⁺ ions, especially Mg²⁺ ions.

A

B

C

D

FIG. 1. Temperature-dependent release of nucleus-bound hCDC47 and hMCM3 proteins from Triton X-100-permeabilized HeLa cell nuclei and its suppression by ATP. A, cells were extracted with mCSK buffer containing 0.5% Triton X-100, and the extracted nuclei were incubated in the same buffer at the indicated temperature for the indicated times. After centrifugation, the supernatant (S) and the pellet (P) fractions were analyzed by immunoblotting with anti-hCDC47 antibodies or anti-PCNA monoclonal antibody. B, Triton X-100-extracted nuclei were incubated in the buffer with or without the addition of ATP (0.1 or 1 mM) at 33 °C for 40 min (or at 0 °C for 40 min without ATP) and processed as in panel A. The samples were also subjected to 15% SDS-PAGE followed by Coomassie Blue staining for detection of core histones. C, selected representative samples in A and B were also immunoblotted with anti-mMCM3 rabbit antisera. The incubation time for the nuclei was 40 min.

tion, is not required for the observed ATP-dependent nuclear binding by hMCMs.

DNA-protein interactions frequently require divalent cations, especially Mg²⁺. Accordingly, we examined the effects of Mg²⁺ on the nuclear association of hMCMs as follows. To avoid undesired nuclear lysis by removal of divalent cations, Triton X-100-extracted nuclei were prepared as usual with 0.5% TX-100 mCSK buffer, which contains 1 mM MgCl₂, and before incubation, they were supplemented with excess EDTA (final 10 mM) or additional MgCl₂. In the presence of EDTA, almost all of hMCMs were released by nuclease digestion. The Triton X-100-extracted HeLa cell nuclei prepared as in panel A, C, effects of MgCl₂, Triton X-100-extracted HeLa cell nuclei prepared as usual with 0.5% TX-100 mCSK buffer, which contains 1 mM MgCl₂, were supplemented with 10 mM EDTA or additional MgCl₂. After incubation at 25 °C for 20 min, the samples were processed as in panel A. D, effects of MgCl₂ in the presence of ATP. Triton X-100-extracted HeLa cell nuclei were prepared as in panel C were supplemented with 1 mM ATP. After incubation in the presence or absence of 10 mM EDTA at 33 °C for 40 min, the samples were processed as in panel A.

Alternatively, Mg²⁺ might not be required in the presence of ATP. These points should be precisely reexamined when a reconstituted system for hMCM-DNA (or chromatin) binding is established.

hMCM Proteins Are Associated with Chromatin—Determination of conditions to stabilize the association of hMCMs with nucleus enabled us to more precisely examine whether they were released by nuclease digestion. The Triton X-100-extracted HeLa cell nuclei were incubated with 1 mM ATP at 25 °C for 30 min under the absence or presence of DNase I, and thereafter the levels of hCDC47 protein in the nuclear pellets and the soluble supernatants were examined by immunoblotting. Without DNase I, almost all of the nucleus-bound hCDC47 remained in the pellet. In contrast, when incubation was with 250 units/ml DNase I, approximately two-thirds of the nucleus-bound hCDC47 was released, and with 1000 units/ml, by which the genomic DNA was digested into less than 400–100 base pairs on average (data not shown), almost all was released (Fig. 3A). Parallel immunoblotting revealed that the nucleus-bound hMCM3 and hdc21 were also liberated by DNase I digestion like hCDC47 (Fig. 3A; data not shown for hdc21). When nuclei were treated with restriction endonuclease HaeIII instead of DNase I, similar release of the nucleus-bound hCDC47 and hMCM3 resulted (Fig. 3B). In both cases, release of the hMCMs by nuclease digestion was accompanied by liberation of core histones (Fig. 3, A and B). We also examined whether the bound hMCMs are released by nuclease di-
Six hMCM Members Are Associated with Chromatin as Heterocomplexes—Although biochemical analyses of mammalian MCM proteins have so far been accomplished mainly using nonionic detergent extractable forms, several lines of investigation of chromatin-bound forms have been attempted with high concentration salt extraction. Using such methods, for example, it has been shown that MCM5 and CDC46 form a complex in the chromatin-bound form as tight as in the soluble one (21, 23, 26). This salt extraction is useful in some instances but might be inappropriate for analyses of interactions among proteins, since only those strong enough to be resistant to high salt concentrations would be maintained. We consider that the above described DNase I-directed solubilization of chromatin-bound hMCMs in the presence of ATP was a viable alternative to salt extraction; it better preserves physical interactions among proteins or between proteins and DNA, although the effects of the DNA remaining after DNase I digestion should be taken into account in the interpretation of results. In the present study, as an initial trial, we focused on physical interactions among the chromatin-bound hMCMs, assessing them by co-immunoprecipitation.

As previously reported, our anti-hCDC47 antibodies specifically detect authentic hCDC47 by immunoblotting and by immunoprecipitation under stringent conditions with radiolabeled precipitation buffer (24). Although the part (amino acids 562–719) of the hCDC47 protein used for antibody preparation is unique, the protein with a region most homologous to this is the protein with a region most homologous to this is hMCM3. On the other hand, the anti-mMCM3 antibodies specifically recognize not only the authentic mMCM3 but also mMCM3 on Western blotting (21, 23). These antibodies, therefore, seemed suitable for aimed co-precipitation analysis. How-
however, to more rigorously establish their specificities, especially with the immunoprecipitation buffer adopted, we prepared in vitro translated hCDC47 and hMCM3. As shown in Fig. 4, reciprocal immunoprecipitation with the anti-hCDC47 or anti-mMCM3 antibodies under the same conditions as used for cellular hMCMs revealed no evidence of any cross-reaction.

The DNase I-released fractions of HeLa cells were immunoprecipitated with anti-hCDC47 antibodies, and the precipitates were washed with 0.1%TX-100mCsK containing 1 mM ATP, subjected to SDS-PAGE, and silver-stained. Anti-hCDC47 antibodies co-precipitated p125, p105, p97, and p90 with p83 corresponding to hCDC47 in the presence of 100 mM NaCl (Fig. 5A). When the NaCl concentration in washing buffer was raised to 500 mM, p125 and p90 were remarkably decreased, but p105 and p97 remained in the anti-hCDC47 precipitates (Fig. 5A). With immunoblotting of the precipitates, the p83 was recognized by anti-hCDC47 as expected, p125 by anti-XMCM2, p97 by anti-mcd21, and p90 by anti-mCDC46 (Fig. 5B). The results for p105 were somewhat complex. Anti-mMCM3 antibodies reacted with p105 in the precipitates after washing with 100 mM NaCl but not after washing with 500 mM NaCl (Fig. 5B), indicating the presence of at least two different proteins, one hMCM3 and another, which is more tightly associated with hCDC47. Recently, hCDC47 has been found to form tight complexes with hcdc21 and a newly identified p105MCM in their soluble forms (33, 34). It is now apparent that p105MCM is a mammalian homologue of Mis5 (39), an MCM family member in fission yeast (11). Therefore, the p105 identified in our system may be hMis5.

To gain further information, the DNase I-released fractions were reciprocally immunoprecipitated with anti-mMCM3 antibodies. As shown in Fig. 6, comparison between anti-hCDC47 and anti-mMCM3 revealed essentially the same co-precipitation patterns for hMCMs, and no significant enrichment of the corresponding antigen was found in the reciprocal immunoprecipitates. As demonstrated by previous studies (21, 26), in the presence of 500 mM NaCl, only hCDC46 was readily co-precipitated with hMCM3 (data not shown). These immunoprecipitation data suggest that the six chromatin-bound hMCM proteins essentially exist in a heterocomplex with the apparent stoichiometric ratio 1:1, in which hCDC47, hcdc21, and hMis5 form one relatively tight complex and hMCM3 and hCDC46 form another. Since a slight, but significantly higher level of DNA compared with the control case was detected in anti-hCDC47 immunoprecipitates (data not shown), we cannot completely eliminate the possibility that the interactions are mediated by residual DNA. However, complexes were also detected in the Triton X-100-extractable fraction and in the fraction released from nonionic detergent-extracted nuclei by ATP-depletion, as described below.

The hMCM Complex Is Dissociated from Chromatin by Heating in the Absence of ATP—We next examined physical interactions among the hMCMs dissociated from chromatin by ATP depletion. The fraction released from nonionic detergent-extracted HeLa cell nuclei by heating in the absence of ATP was immunoprecipitated with anti-hCDC47 or anti-mMCM3 antibodies, and the precipitates were washed with 0.1%TX-100mCSK, subjected to SDS-PAGE, and silver-stained. Anti-hCDC47 antibodies co-precipitated p125, p105, p97, and p90, with p83 (Fig. 7A). With immunoblot analyses of the anti-hCDC47 precipitate, p125 was recognized by anti-XMCM2, p90 by anti-mCDC46, and p105 by anti-mMCM3 (Fig. 7B). The relative levels of the co-precipitated hMCM3 and hCDC46 to hCDC47 were slightly decreased in this fraction as compared with the chromatin-bound case. Therefore, most hMCMs dissociated from chromatin by ATP depletion appeared to remain in complexes. Unlike the DNase I-released fraction, however, differ-
ent precipitation profiles were obtained with anti-hCDC47 and anti-mMCM3 antibodies; namely, anti-mMCM3 antibodies failed to precipitate the complex efficiently, co-precipitating only p90, p125, and additional p126 with p105 hMCM3, with apparently equivalent stoichiometry (Fig. 7A). Immunoblot analyses demonstrated anti-mCDC46 antibodies to recognize p90 as expected, but anti-XMCM2 recognized neither p125 nor p126 (Fig. 7B). The nature and significance of these latter two is currently unknown. One interpretation of the discrepancy between the two immunoprecipitates is that the anti-mMCM3 antibodies contain those targeting epitopes on hMCM3 contributing to binding between the hMCM3-hCDC46 complex and the hCDC47-hcdc21-hMis5 complex, enhancing dissociation of the two complexes. The possibility that the hMCM3-hCDC46 complex was dissociated preferentially from nuclei by ATP depletion can be ruled out, since the dissociation rate of hMCM3 was similar to that of hCDC47 (see Fig. 1). In the chromatin-bound form in the presence of ATP, the epitopes might be masked conformationally or by other protein(s). Alternatively, even after dissociation by the antibodies, the two complexes might be linked together by factors such as other proteins or DNA protected from DNase I.

The hMCM Complex in the Triton X-100-extractable Fraction—We also examined complex formation by the nonionic detergent-extractable hMCMs by immunoprecipitation. The Triton X-100-extractable fractions of HeLa cells were immunoprecipitated with anti-hCDC47 antibodies, and the precipitates were washed with 0.1%TX-100mCSK containing 100 mM or 500 mM NaCl. The precipitates were subjected to SDS-PAGE followed by silver staining, showing similar co-precipitation profiles as with the DNase I-released fraction (Fig. 8). The identity of each hMCM except hMis5 was confirmed by immunoblotting (data not shown). The relative levels of higher mobility forms of hMCM2 and retarded forms of hcde21 in the precipitate appeared increased in comparison with those in the chromatin-bound form, in agreement with previous findings that these forms result from hyperphosphorylation and can be readily extracted with nonionic detergent (21, 22). These results indicate that soluble hMCMs also exist, at least partly, as hetero-complexes, in which hCDC47, hcde21, and hMis5 similarly form a relatively tight complex. On the other hand, the anti-

**Fig. 6.** Both anti-hCDC47 and anti-mMCM3 antibodies precipitated chromatin-bound hMCM heterocomplexes without significant enrichment of the cognate antigen. A, DNase I-released fractions obtained as described in Fig. 5 were immunoprecipitated with a control rabbit immunoglobulin (lane 1), anti-mMCM3 (lane 2), or anti-hCDC47 (lane 3) antibodies. The precipitates were washed with buffer containing 100 mM NaCl and then separated by 10% SDS-PAGE followed by silver staining. B, the precipitates were analyzed by immunoblotting with anti-hCDC47 or anti-mMCM3 antibodies. Lane 1, control precipitates; lane 2, anti-mMCM3 precipitates; lane 3, anti-hCDC47 precipitates.

**Fig. 7.** Release of the hMCM complex from chromatin by ATP depletion. A, Triton X-100-extracted HeLa cell nuclei were incubated in the absence of ATP at 25 °C for 30 min. The supernatants were immunoprecipitated with a control rabbit immunoglobulin (lane 1), anti-hCDC47 (lane 2) or anti-mMCM3 (lane 3) antibodies. The precipitates were washed with buffer containing 100 mM NaCl and then separated by 10% SDS-PAGE followed by silver staining. B, the precipitates were analyzed by immunoblotting with anti-hCDC47, anti-mMCM3, anti-mCDC46, or anti-XMCM2 antibodies. Lane 1, control precipitates; lane 2, anti-hCDC47 precipitates; lane 3, anti-mMCM3 precipitates.

**DISCUSSION**

**hMCM Complexes**—Since sequence requirements for replication origins appear to differ among budding yeast, egg extract, and mammalian somatic cells, MCMs might function in distinct ways in each case. Whereas biochemical studies of physical associations among mammalian MCMs have been so far carried out mainly using chromatin-unbound soluble forms (21, 23, 26, 33, 34), those playing a role in allowing the initiation of replication may more likely be the nucleus-bound forms. In the present study, we have therefore focused attention on chromatin-bound hMCMs solubilized by DNase I in the presence of ATP. Our newly devised approach is partly analogous to the UV or formaldehyde cross-linking-immunoprecipitation techniques applied for analyzing protein-DNA interactions in vivo (40, 41). The data obtained indicate that six hMCM family members associate with chromatin almost exclusively as a heterocomplex, in which hCDC47, hcde21, and hMis5 form one relatively tight complex, while hMCM3 and hCDC46 form...
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FIG. 8. The presence of hMCM complexes in Triton X-100-extractable fractions. HeLa cells were extracted with nCSK buffer containing 0.1% Triton X-100 to obtain soluble hMCMs, and the lysates were immunoprecipitated with control rabbit immunoglobulin (lanes 1 and 4), anti-hCDC47 (lanes 2, 3, and 5) or anti-mMCM3 (lane 6) antibodies. The precipitates were washed with buffer containing either 100 mM (lanes 1, 2, 4, 5, and 6) or 500 mM NaCl (lane 3) and then separated by 10% SDS-PAGE followed by silver staining.

another.

It has been shown using gel filtration in the presence of glycerol or by glycerol gradient centrifugation that soluble fraction hMCMs reside in a 500–600-kDa complex (26, 33, 34). However, the exact composition of the complex remains to be elucidated. Interactions between MCM3-CDC46 and CDC47-cdc21-Mis5 complexes have so far not been detected with standard immunoprecipitation procedures (23, 26, 33, 34). In contrast, the present immunoprecipitation assay also demonstrated the heterocomplex consisting of six hMCMs in the Triton X-100-soluble chromatin-unbound fraction. Our conditions may thus be more stabilizing than those applied by the standard method. It has been reported that glycerol stabilizes the 500–600-kDa hMCM complex during gel filtration (33). Taking into account our findings and the above described previous findings, it seems likely that hMCMs play a role in DNA replication as heterohexamers associated with chromatin. Whether this is also the case for the egg extract or yeast systems is unclear, although a 500–600-kDa MCM heterocomplex has also been detected in the chromatin-unbound fraction in both cases (16, 30, 31, 32). It is furthermore conceivable that the hMCM complexes undergo cell cycle modifications such as association-dissociation with chromatin or phosphorylation, but the possibility still remains that there is a change in the complex formation profile during the cell cycle. This could be clarified by examining synchronized cell populations with the methods described here. On the other hand, it is clearly of interest that any association of chromatin-bound complexes with other protein(s) be clarified, and co-immunoprecipitation studies might be one fruitful approach to achieving this end.

The hMCM Complex Chromatin Association—In the present study, we showed that under three different conditions, nucleosome-bound hMCMs are released depending on nucleosome digestion. To date, a number of authors have investigated the effects of DNase I treatment on the nuclear association of mammalian MCMs. Kimura et al. (21) reported no release of nucleus-bound mMCM3 under their conditions. Burkhardt et al. (26) described approximately half of the bound hMCM3 as liberated, although details of their assay were not given. Todorov et al. (22) found that digestion of HeLa cell nuclei with DNase I released the bound hMCM2 without affecting the core histones, in direct contrast to our present results. Although it is difficult to directly compare findings obtained under different experimental conditions, a major advantage with our system was the stabilization of chromatin binding of the hMCM complex observed with ATP. However, it is still unclear whether the complex binds to DNA directly or requires the mediation of another protein(s) and, if directly, which hMCMs participate in the binding. Recently, it was reported that hMCMs can bind to histone H3 in vitro (42). However, in anti-hCDC47 immunoprecipitates from the DNase I-released fractions, core histones have not been detected in comparable stoichiometry with hMCMs as yet.2 On the other hand, it is also uncertain whether specific DNA sequences are involved in the binding. In this regard, we have observed that HaeIII-PvuII-digested DNA fragments can be co-precipitated by anti-hCDC47 only in the presence of ATP and not by control antibodies.2

Possible Involvement of ATP in the hMCM Complex Chromatin Association—The possibility that chromatin binding of hMCMs is stabilized by ATP is intriguing. The ability of AMP-PNP to replace ATP indicates that reactions requiring hydrolysis of the ATP β-γ bond like protein phosphorylation or ATPase dependent steps are not required for the observed effect. Rather, ATP might act like a co-factor that enhances DNA binding as is the case with several replication-related DNA-binding proteins such as SV40 or polyomavirus T-antigen; the binding of the T-antigen to the origin is enhanced not only by ATP but also by AMP-PNP (43, 44). Since it is unclear whether the hMCMs bind to DNA directly or via other DNA-binding protein(s), we cannot even speculate as to the identity of candidate protein(s) affected by ATP. One possibility, however, is that the hMCM complex binds to DNA directly in an ATP-dependent manner. It is known that hMCMs have putative ATP-binding and ATPase motifs in their conserved regions (45), but we have yet failed to detect either in immunoprecipitated hMCM complex preparations. Another possibility is that the hMCM complex binding is through other protein(s) that associate with DNA in an ATP-dependent manner. One attractive candidate for such a protein(s) might be the putative human homologue of budding yeast origin recognition complex (46), since this multi-protein complex binds to ARS in an ATP-dependent manner and interacts genetically with MCMs in budding yeast (47, 48). In contrast to the case with the T-antigen, however, origin recognition complex DNA binding is not stimulated by AMP-PNP (47). In any case, our results do provide a strong indication that the hMCM complex association with chromatin in vitro is dependent to some extent on ATP. Although the exact role of ATP in the chromatin association of hMCMs and its generality in other systems such as egg extracts or yeast remains to be determined, its effect should be taken into account in future analyses of MCM-DNA interactions.

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