Minichromosome maintenance (MCM) proteins play essential roles in eukaryotic DNA replication, but their biochemical properties remain to be determined. We detected in HeLa cell extracts six proteins, CDC47, CDC46/MCM5, Cdc21, P1/MCM3, Mis5, and BM28/MCM2, by their binding to a specific antibody and by partial sequencing. The human homologs of the MCM2 (BM28), Mis5, Cdc21, and CDC47 proteins were tightly bound to a histone-Sepharose column and purified to near homogeneity, whereas the P1/MCM3 and CDC46/MCM5 proteins passed through. Among the four core histones, the human BM28/MCM2, Mis5, Cdc21, and CDC47 proteins had high affinity for histone H3. Immunoprecipitation with anti-Cdc21 antibody revealed that these four MCM proteins form complexes. These results are consistent with the findings that MCM proteins bind with chromatin in vivo.

In eukaryotic cells, DNA replication initiates from multiple sites in chromosomes during S phase, which are called replication origins. In Saccharomyces cerevisiae, several proteins that are required for the initiation of DNA replication have been identified. Origin recognition complex (ORC)1 proteins (1) that bind to the essential origin sequence are involved in DNA replication (2–5). MCM proteins, which include MCM2, MCM3, CDC46/MCM5, CDC47, and CDC54 (6–9), are essential for cell growth and are required for DNA replication. Genetic analyses indicate that they have distinct roles in DNA replication and that they interact (7, 8). Interactions between the components of ORC with MCM proteins have also been described (5, 10). In Schizosaccharomyces pombe, Cdc21 (11) and Mis5 (12) MCM proteins in addition to Nda1/MCM2 and Nda4/CDC46 (13) have been identified. Homologues of these MCM proteins are found in various organisms and Xenopus MCM3 (14–17), Drosophila MCM2 (18) and Cdc21 (19), mouse P1/MCM3 (20), and human BM28/MCM2 (21) proteins also play essential roles in cellular DNA replication. However, it remains to be analyzed how the MCM proteins are involved in this process.

Since MCM proteins have the domains required for DNA-dependent ATPase activity in central regions (22), they may function as the DNA helicase that is required for DNA unwinding during DNA replication, although human BM28/MCM2 (23), Cdc21, CDC47, and MCM3 (24), and mouse MCM3 (20) proteins are not co-localized in replication foci. P1/MCM3 protein may be a component of the DNA polymerase α holoenzyme (25). Immunocytochemical analyses suggest that human BM28/MCM2 (23) and mouse P1/MCM3 (20, 26) proteins bind with the nuclear structure in G1 phase and detach from it during DNA replication. The sensitivity to nuclease suggests that both human BM28/MCM2 (23) and S. cerevisiae MCM3 (27) proteins bind with chromatin. We detected MCM proteins in HeLa cell extracts by immunoblotting, using antibodies against a conserved domain of MCM proteins, and found that MCM protein complexes containing BM28/MCM2, Mis5, Cdc21, and CDC47 proteins have high affinity for histone H3.

EXPERIMENTAL PROCEDURES

Proteins and Serum—Histone pairs of H2A/H2B and H3/H4 were purified from HeLa cells as described by Simon and Felsenfeld (28). H3 and H4 histones were purified from mouse FM3A cells. They were separated by HPLC using a reverse phase C18 column. The histones were eluted by a linear gradient from 20 to 60% acetonitrile containing 0.1% trifluoroacetic acid. H4 and H3 histones were eluted from the column by approximately 45 and 50% acetonitrile, respectively. Separation of these two histones was confirmed by electrophoresis in a 15% polyacrylamide gel containing sodium dodecyl sulfate (29). Histones other than H4 were diazylated with 0.1 mM NaHCO₃, pH 8.5, and cross-linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech Inc.) (30). Since H4 histone was insoluble in the buffer, it was diazylated against 0.1 M potassium phosphate, pH 7.5, and then NaHCO₃, pH 8.5, was added at the final concentration of 0.1 M before cross-linking. Histones (0.3–0.5 mg) were cross-linked to 1 ml of Sepharose 4B. Rabbit antiserum against the peptides (VVCIDEFDKMSDMDRTA) from mouse MCM proteins that had been linked to keyhole limpet hemocyanin was raised (31). The specific antibodies (rabbit) against mouse P1/MCM3, CDC46/MCM5, and Cdc21 proteins were raised as described elsewhere (20, 32). A carboxyl-terminal region (amino acid 683–862) of mouse Cdc21 protein was produced in Escherichia coli as a glutathione S-transferase fusion protein (Pharmacia). The rabbit antibodies against the protein were raised and they were affinity-purified. Anti-Cdc21 antibody beads (2 mg/ml) were prepared by cross-linking the antibodies to cyanogen-activated Sepharose 4B. The beads were also conjugated with rabbit immunoglobulin (5 mg/ml) that had been purified from preimmune serum by a protein A column. For immunoblotting, the proteins in a 10% polyacrylamide gel were transferred to a nylon membrane (Immobilon-F, Millipore) and then incubated for 1 h at 37 °C in TBS (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 5% horse serum (Figs. 1–3, 5, and 7) or skim milk (Fig. 8). The membrane was incubated for 1 h with anti-MCM protein antibody (rabbit antibody) diluted with the solution described above. After washing with TBS containing 0.1% Triton X-100, the membrane was incubated for 1 h with diluted peroxidase-conjugated anti-rabbit antibody (goat antibody, Bio-Rad). Proteins were visualized using a peroxidase detection kit (HRP1000, Konica) (Figs. 1–3, 5, and 7) or a chemiluminescent substrate system (Pierce) (Fig. 8). Logarithmically growing HeLa cells and those in mitotic phase were kindly provided by Keiji Kimura (33).

Purification of MCM Proteins—HeLa cells were cultured in spinner
bottles until late-log phase. After the cells (1 × 10^10 cells) were homogenized in hypotonic buffer, 5 mM NaCl was added to a final concentration of 0.2 M, and 1 mg of lysyl endopeptidase (Acromabacter, Wako) in 8% acetic acid, 20 mM Tris-HCl, pH 8.5. After centrifugation, the supernatant was saved. The beads were washed four times with phosphate-buffered saline containing 0.05% Nonidet P-40, and proteins bound to the beads were recovered by eluting twice with 20 μl of 0.1 M glycine, pH 2.5, and 0.15 M NaCl. The solution was neutralized and then subjected to electrophoresis.

**Other Methods**—Proteins in the gel were stained with Coomassie Blue or silver (silver stain kit, Wako). The protein concentration was determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard.

### RESULTS

#### Detection of MCM Proteins in 0.2 M NaCl-soluble and 2 M NaCl-soluble Extracts of HeLa Cells—Six MCM proteins of MCM2, MCM3, CDC46/MCM5, CDC47, Cdc21, and Mis5 have been identified in eukaryotic cells (Table I) (32, 36, 37). They have a conserved region in the central part of the proteins (12, 21, 31, 37) where the domains required for DNA-dependent ATPase activity are present (22). Antibodies against a peptide from a highly conserved region of MCM proteins were obtained by immunizing rabbits with a synthetic peptide from the region (31). The antibodies recognized several proteins ranging from 80 to 125 kDa in addition to smaller proteins in 0.2 M NaCl-soluble whole cell extracts of HeLa cells on an SDS-polyacrylamide gel, which was analyzed by immunoblotting (Fig. 1, lane 1). The extracts were fractionated by ammonium sulfate precipitation followed by DE52 column chromatography. Immunoblotting showed that the MCM-related proteins of higher molecular mass were bound to the DE52 column, and they were eluted from the column near 0.2 M NaCl (Fig. 1). In the pooled DE52 fractions (fractions 9–15), at least three (86-, 100-, and 125-kDa) proteins, all of which were recognized by the antibodies to MCM proteins, were detected (Fig. 2A). The specific antibodies to mouse P1/MCM3 protein recognized a 100-kDa protein, and those to mouse Cdc21 protein recognized 86- and 100-kDa proteins on immunoblots. Antibodies to mouse CDC46/MCM5 did not detect any proteins, but a 90-kDa protein in the DE52 fraction was detected by the antibodies (as shown in Fig. 7). A 90-kDa protein recognized by the antibodies against the conserved portion of MCM protein was detected in the fractions eluting at about 0.15 M NaCl in the DE52 column chromatography, but the protein was not detected in the fractions eluting at a higher NaCl concentration (Fig. 1). It appears that the CDC46/MCM5 protein was present in the pooled DE52 fraction, but the binding of the anti-MCM protein antibodies to the CDC46/MCM5 protein was interfered with by a protein(s) in the pooled fraction that migrates at a similar position to the 90-kDa CDC46/MCM5 protein in the SDS-polyacrylamide gel under the conditions described in Fig. 2A.

The nuclear precipitate remaining after the extraction with 0.2 M NaCl was treated with 2 M NaCl, and the extracted proteins were fractionated by hydroxyapatite column chromatography. Proteins of 90, 100, and 125 kDa were detected in the
The proteins in HeLa cell extracts (lane 1), ammonium sulfate fraction (lane 2), and the DE52 fractions (lanes 3–25) were electrophoresed on a 10% polyacrylamide gel and analyzed by immunoblotting by using antibodies against the conserved portion of MCM protein. The proteins passed through the DE52 column were electrophoresed in lanes 3 and 4, and those eluted from the column by increasing the concentration of NaCl were run in lanes 5–25. The concentration of NaCl, which was determined by examining the conductivity of the eluate, is indicated at the top of the gel. The molecular mass was determined by comparison with marker proteins (Bio-Rad, high range).

The fractions containing the MCM proteins that were obtained from 2 M NaCl-soluble whole cell extracts were analyzed by histone-Sepharose column chromatography (Fig. 2). The 100-kDa protein in the purified fraction was recognized by antibodies against mouse Cdc21 and CDC46/MCM5 proteins, respectively. These results indicated that the 125-, 103-, 100-, and 86-kDa proteins correspond to P1/MCM3 and CDC46/MCM5 proteins. A 86-kDa protein in whole cell extracts that was recognized with the anti-mouse Cdc21 antibody did not bind with the histone column. Immunoblotting showed that P1/MCM3 protein in the DE52 fraction (Fig. 2A) passed through the histone-Sepharose column (data not shown, see Fig. 5C).

The partial amino acid sequences of the proteins bound to the histone-Sepharose column were determined after digesting the proteins with lysyl endopeptidase (Fig. 4). Two peptides obtained from the 125-kDa protein were localized in boxes A (peptide 1) and D (peptide 2) (31) of human BM28/MCM2 protein (21) (Fig. 4A). One peptide from the 86-kDa protein was identified in box A of human CDC47 (P1.1/MCM3) protein (38) that has been partially cloned (31), and another peptide was almost matched to a peptide of mouse CDC47 protein (39). Four of five peptides from the 103-kDa protein were localized in a rat intestinal crypt protein that has been partially cloned (40) (Fig. 4B). Homology of the rat protein to the central region of S. pombe Mis5 protein was greater than that to the human MCM proteins, including MCM2/BM28, P1/MCM3, CDC46/MCM5, Cdc21, and CDC47 (Fig. 4B and data not shown). These results suggested that the 125-, 103-, 100-, and 86-kDa proteins correspond to human BM28/MCM2, Mis5, Cdc21, and CDC47 proteins, respectively. These results indicated that the human homologs of the MCM2, Mis5, Cdc21, and CDC47 proteins have a high affinity for histones, but P1/MCM3 does not. Immunoblotting showed that about 400 μg of MCM protein were precipitated in the ammonium sulfate fraction (0.3% of total protein), and the final histone-Sepharose fraction contained about 50 μg of the protein.
loaded onto the histone-Sepharose column, these two MCM proteins passed through the column (data not shown).

**Binding of MCM Proteins with Histone H3**—To understand the interaction of the MCM proteins with histones, the MCM proteins of the DE52 fraction were loaded onto a histone H2A/H2B or a H3/H4 column, instead of the histone-Sepharose column containing four core histones (Fig. 5). Proteins of 86, 100, 103, and 125 kDa were bound to the H3/H4 column and eluted near 1 M NaCl (Fig. 5B). The composition was similar to that in the fractions purified by the histone-Sepharose column chromatography (Fig. 3). All four proteins were recognized by the antibodies against the conserved portion of MCM proteins, and the 100-kDa protein was recognized by anti-Cdc21 antibodies (data not shown). However, the 102-kDa protein passed through the H3/H4 column, and it was recognized by the spe-
specific antibodies against P1/MCM3 protein (Fig. 5C). These results are essentially the same as those obtained after histone-Sepharose column chromatography. No MCM proteins were detected in the bound fractions of H2A/H2B column chromatography (Fig. 5A), but they passed through the column (Fig. 5C). NAP-I was bound to the H2A/H2B but not to the H3/H4 column, since it has a higher binding affinity for H2A/H2B than for H3/H4 (41). Next, the DE52 fraction was loaded onto a histone H3 or a H4 column (Fig. 6). The 86-, 100-, 103-, and 125-kDa proteins bound to the H3, but not to the H4 column. They were mainly eluted from the H3 column by 0.5–1 M NaCl; the concentration was slightly lower than that required for the elution from the H3/H4 column. These results indicated that the human homologs of the MCM2, Mis5, Cdc21, and CDC47 proteins specifically interact with histone H3. However, the possibility that these MCM proteins also interact with H4 histone cannot be excluded.

**Complex Formation of MCM Proteins**—To know the interaction of MCM proteins, the fraction containing MCM proteins were immunoprecipitated with anti-Cdc21 antibody beads (Fig. 7). Four MCM proteins of BM28/MCM2, Mis5, Cdc21, and CDC47 in the purified fraction were precipitated with the beads conjugated with anti-Cdc21 antibody, but not with those conjugated with control rabbit antibody (Fig. 7A). The anti-Cdc21 antibody beads also precipitated four proteins of 125, 100, 90, and 86 kDa from the DE52 fraction, which was analyzed by immunoblotting (Fig. 7B). The 125-kDa protein most probably corresponds to BM28/MCM2 and the 86-kDa protein to CDC47. Binding of specific antibodies showed that Cdc21 and P1/MCM3 proteins of about 100 kDa were precipitated with the anti-Cdc21 antibody beads, and the 90-kDa CDC46/MCM5 protein was also precipitated (Fig. 7C). Three MCM proteins of Mis5, Cdc21, and P1/MCM3 may migrate at the same position under these conditions. Therefore, it was suggested that six MCM proteins in DE52 fraction are all precipitated by the Cdc21 antibodies. In addition to some portion of 125-kDa MCM2 protein, however, a 100-kDa protein was not precipitated with the anti-Cdc21 antibodies. The 100-kDa protein was recognized with anti-P1/MCM3 antibodies, but not with anti-Cdc21 antibodies by immunoblotting (data not shown). These results suggest that BM28/MCM2, Mis5, Cdc21, and CDC47 proteins, which were tightly bound with histone H3, form complexes, and P1/MCM3 and CDC46/MCM5 proteins in DE52 fraction are also associated with these complexes. When the purified MCM proteins were fractionated by sucrose gradient centrifugation in the buffer containing 0.5 M NaCl, a major peak of MCM proteins, where the BM28/MCM2, Mis5, Cdc21, and CDC47 proteins were present, was detected at 11 S (data not shown). These data support the conclusion that they form complexes.

Human BM28/MCM2 (23) and mouse P1/MCM3 (20) proteins are phosphorylated as DNA replication proceeds. Phos-
England Biolabs) in the presence of 2 mM MnCl₂ at 30°C for 30 min. The protein in the gel was almost the same as that of MCM2 and CDC46, only the 125-kDa protein was detected. The mobility of the top of the gel.

Phosphatase is indicated by + at the top of the gel. Treatment with phosphatase is indicated by + at the top of the gel. The conserved portion. Treatment with phosphatase is indicated by + at the top of the gel.

Phosphorylated human BM28/MCM2 protein migrates faster than underphosphorylated forms of the protein in SDS-polyacrylamide gel (23). To know the phosphorylation state of MCM2 protein in the purified fractions, the mobility of MCM2 protein in the purified fraction was compared with that of MCM2 protein in extracts prepared from logarithmically growing HeLa cells and also mitotic cells (Fig. 8). Two bands of 125 and 120 kDa, which are most probably MCM2 proteins, were detected in the extracts from growing cells in addition to the MCM proteins of smaller molecular mass, and several bands ranging from 135 to 120 kDa were detected in the extracts from mitotic cells. After incubating these extracts with λ phosphatase, only the 125-kDa protein was detected. The mobility of the protein in the gel was almost the same as that of MCM2 protein in the purified fraction. These results suggest that the MCM2 protein in the purified fraction is an underphosphorylated form.

DISCUSSION

We showed that human homologs of MCM2 (BM28), Mis5, Cdc21, and CDC47 proteins bind histone. The four MCM proteins, which form complexes, specifically bind histone H3 among four core histones. P1/MCM3 and CDC46/MCM5 proteins did not bind histone, but these two MCM proteins from 0.2 M NaCl-soluble whole cell extracts are associated with the above MCM protein complexes. Both human BM28/MCM2 (23) and mouse P1/MCM3 (20, 26) proteins are bound with nuclear structures in the G₁ phase. The sensitivity to nuclease suggests that human BM28/MCM2 (23) protein binds with chromatin. The specific binding of MCM proteins to histone H3 may explain their binding to chromatin in vivo. It remains to be determined whether or not all of the four MCM proteins have a binding affinity for histone H3. Although some portion of BM28/MCM2 protein was not precipitated with anti-Cdc21 antibodies (Fig. 7B), almost all of the protein bound with histone (Fig. 5C), suggesting that BM28/MCM2 protein itself can bind with histone H3. Musahel et al. (42) have reported that human Cdc21 protein forms a stable trimeric complex with CDC47 and 105-kDa MCM proteins, and BM28/MCM2 protein is loosely associated with the trimeric Cdc21 complex. Immunoprecipitation with anti-Cdc21 antibodies (Fig. 7) supports these findings, and the 105-kDa MCM protein probably correlates to the 103-kDa protein of human Mis5 protein in our study. Among six MCM proteins, the behavior of the P1/MCM3 and CDC46/MCM5 differed from that of other MCM proteins, which may be consistent with the findings that these proteins form complexes in mouse cells (32) and in human nuclear extracts (43). In addition, two MCM complexes of 600 kDa, one that contains CDC46 and one that appears to contain both MCM2 and Cdc21 proteins, have been identified in Drosophila extracts (44). The sensitivity to nuclease suggests that S. cerevisiae MCM3 protein also binds with chromatin (27). MCM3 protein may bind chromatin by interacting with other MCM proteins, since MCM3 protein complexes with MCM2 and Cdc21 proteins in Xenopus egg extracts (45), and P1/MCM3 and CDC46/MCM5 proteins in DE52 fraction interacted with the complexes containing other MCM proteins (Fig. 7). P1/MCM3 and CDC46/MCM5 proteins may be released from the MCM protein complexes under the conditions (0.3 M NaCl) where the MCM proteins in the DE52 fraction were loaded onto a histone-Sepharose column (Fig. 5). All of the MCM2 (BM28), Mis5, Cdc21, and CDC47 proteins have a putative Zn-binding motif (37), which may play an important role in forming the complexes.

The human BM28/MCM2 (23), Cdc21, CDC46 and MCM3 (24), and mouse MCM3 (20) proteins are not concentrated at the replication forks, since the localization of the MCM proteins in the cells is not coincident with those of the replication proteins of RPA (HSSB) and PCNA that is an accessory protein of DNA polymerase δ. These in vivo findings suggest that they are not a DNA helicase that is present at the replication forks. Complexes containing BM28/MCM2, Mis5, Cdc21, and CDC47 proteins have a high affinity for H3 histone, which forms a tetramer complexed with histone H4 and occupies the central region in the nucleosome structure (46). These two histones play a crucial role in forming a nucleosome structure (47, 48). Histone H2A/H2B dissociates from chromatin near 1 M NaCl and H3/H4 near 1.5 M NaCl (49). The four MCM proteins elute mainly from the histone H3/H4 column at 1–1.6 M NaCl (Fig. 5). These results suggest that the affinity of the MCM proteins to histone H3 seems to be comparable to that of histone H3 to DNA. Therefore, the MCM proteins may play a role in DNA replication by changing nucleosome structures. The activities of nucleosome disruption and concomitant transcription factor binding, which are dependent on ATP hydrolysis, have been reported in a transcription complex of SWI/SNF (50, 51) that may interact with histone H3 and H4 (52). Three possibilities can be considered for the physiological meanings of the high affinity of MCM proteins to histone H3. First, the MCM proteins may disassemble nucleosome structures near the replication origin through interaction with the origin binding proteins. Thereby, the other replication proteins can be assembled to the origin. Consistent with this notion, interaction between the components of ORC and MCM protein has been suggested in S. cerevisiae (5, 10). However, MCM are abundant proteins and are estimated to present at 10⁶–10⁷ molecules/cell (23, 53). They may be present in other chromosomal regions than in the replication origins. Second, the MCM proteins, which have affinity for histone, are required for progression of the replication forks by displacing the nucleosome structures of the unreplicated DNA (23). Krude and Knippers (54) have reported that re-replication of the SV40 minichromosome in vitro is inhibited at the stage of chain elongation, after the chromosome is constructed in a DNA replication-dependent manner. They have interpreted this finding to mean that the specific structure of post-replicative chromatin interferes with the progression of the replication forks. MCM proteins may relieve the structural constraints upon replication during elongation.
However, the second notion may not be consistent with the findings that microinjection anti-BM28 antibody inhibits the initiation of DNA replication but not the progression of DNA replication (21). Third, MCM proteins may disassemble nucleosome structures of chromatin, not specifically in the origin region, but to generate negative DNA superhelicity, since the negative superhelicity is embedded in the nucleosome structure. In topologically fixed chromosomal domains, the torsional stress of the negative superhelicity can be propagated to the origin regions, they may be derived from chromatin-bound forms. It and CDC47 proteins from 0.2M NaCl-soluble whole cell extracts. Although we purified BM28/MCM2, Mls5, Cdc21, and CDC47 proteins from 0.2 M NaCl-soluble whole cell extracts, they may be derived from chromatin-bound forms. It may be important to examine the affinity to histone H3 at the molecular level and to address the physiological significance of this interaction.

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