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Expression, Nuclear Localization and Interactions of Human MCM/P1 Proteins

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We report here the comparative analysis of human Mcm/P1 proteins (HsMcm2, -3, -5 and -7), including a characterization of their mutual interactions, cell cycle dependent expression and nuclear localization during the cell cycle and the quiescent state. The mRNA levels of these genes, which undergo cell cycle dependent oscillations with a peak at G1/S phase, may be regulated by E2F motifs, two of which were detected in the 5′ upstream region of the HsMCM5 gene. In contrast, the protein levels of these Mcm proteins were found to remain rather constant during the HeLa cell cycle. However, their levels gradually increased in a variable manner as KD cells progressed from G0 into the G1/S phase. In the G0 stage, the amounts of HsMcm2 and -5 proteins were much lower than those of HsMcm7 and -3 proteins, suggesting that they are not present in stoichiometric amounts, and that only a proportion of these molecules actively participate in cell cycle regulation as part of Mcm/P1 complexes.

The replication of DNA occurs once, and only once per cell cycle in eukaryotes. Blow and Laskey (1) attempted to explain this tight control by proposing the existence of a hypothetical licensing factor that would bind to chromatin during mitosis to permit DNA replication during the ensuing S phase in *Xenopus* egg extracts. In the budding yeast, *Saccharomyces cerevisiae,* minichromosome maintenance (*mcm*) mutants and some cell division cycle (*cdc*) mutants (2) have been put forward as putative candidate molecules for the replication licensing factor. Actually, replication licensing activity was recently identified in a complex containing MCM/P1 family proteins in *Xenopus* oocytes (3-5).

The MCM genes are widely conserved, as homologues have been reported in fission yeast, insects, plants, frog, mouse and human (6,7). Because the first homologue to be identified in higher eukaryotes was the mouse P1 protein, it has been suggested that proteins that share extensive sequence similarity within a central 200-amino acid region be referred to as MCM/P1 family proteins (6). The MCM/P1 family genes have been classified into six related groups by phylogenetic analysis, and it appears that each organism harbors six kinds of MCM/P1 gene (6,7). To avoid any confusion that might arise from the use of distinct names for MCM genes identified in different organisms, we will employ the unified nomenclature of Chong et al. (6) where each MCM gene is given a number, resulting in the sequence *MCM2, -3, -4, -5, -6,* and *MCM-7.*

In mammals, cDNA sequences for five Mcm/P1 family proteins have been reported (8), and it was shown that human Mcm2 and Mcm5 proteins form a complex (9). Microinjection of antibodies against murine Mcm3 or human Mcm2 inhibited DNA synthesis suggesting a pivotal role for these proteins in DNA replication (10,11). Human Mcm4 (a Cdc21 homologue) has also been shown to form a stable trimeric complex with two other human Mcm proteins, p85Mcm (Mcm7) and p105Mcm (12). Human Mcm7 protein (a Cdc47 homologue) was found to be located in the nuclei of interphase cells but to be evenly distributed throughout the cell during mitosis (13, 14). However, the relative amount of these proteins during the cell cycle and their behavior during the quiescent state, has not been clarified, even though such data is essential for our understanding of their physiological role. Here we report the characterization of these Mcm/P1 genes and their gene

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products, including the analysis of their expression, nuclear localization during the cell cycle and quiescent state, and their mutual interactions. We have also assigned the chromosomal location of the *HsMCM5* gene, which has remained elusive heretofore, to 22q13.1.

MATERIALS AND METHODS

Preparation of GST-fusion constructs. To create the in-frame junction to the GST (glutathione S transferase)-fusion vector, a synthetic linker was inserted into the pGEX-2T vector (Pharmacia, Uppsala, Sweden), in order to allow the cDNA inserts to be fused in-frame to the vector via the *Asc* I-*Not* I sites. To obtain the cDNA insert for HsMcm7 carrying in-frame *Asc* I-*Not* I sites, a set of oligonucleotides around the initiation codon (plus *Asc* I site) and the termination codon (plus *Not* I site) of HsMcm7 were synthesized (Primers N and C in Figure 2A) and used as primers for PCR with the cDNAs as substrates. The identity of each gene was confirmed by DNA sequencing of four independent clones, and the plasmid DNA without mismatched DNA sequence was selected, cut with *Asc* I and *Not* I, and the generated cDNA inserts were incorporated into the GST-vector. A similar strategy was employed to create GST-fusion proteins of HsMcm2, -3, and -5. Plasmid DNA containing the GST-fusion genes were introduced into *E. coli* cells (*PR745*) and the cells were grown at 20°C to minimize protein degradation. Expression from the *lac* promoter was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

Immunoprecipitation and Western blot analyses. Immunoprecipitation and Western blot analyses were performed as described before (15). Briefly, KD or HeLa cells were collected and lysed in Lysis buffer (50mM Tris-HCl[pH.7.5], 250 mM NaCl, 0.1% NP-40) supplemented with protease inhibitors and phosphatase inhibitors (10 mM $Na_4P_2O_7$, 10mM NaF, 2mM sodium vanadate and 1mM EGTA). Then, the immunoprecipitated cell extract or whole cell extract (10μg) was separated by 10% SDS-PAGE, and transferred onto an Immobilon membrane (Millipore). The membrane was probed with relevant antibodies in TBST buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% tween 20). Immunoreactive protein bands were visualized by chemiluminescence reagents (Dupon, U.S.A.).

Cloning of the 5′ regulatory region of the HsMCM5 gene. The 5′ franking region of *HsMCM5* gene was cloned using Promoter Finder™ DNA walking kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's protocol. Briefly, a set of nested oligonucleotides upstream of the ATG codon of *HsMCM5* cDNA was synthesized, and used for the PCR reaction with a pool of adapter-ligated genomic DNA fragments as template. The generated PCR product was ligated to the plasmid vector, and the plasmid DNA from four independent clones was isolated. The almost identical DNA sequence of these four clones as determined by a DNA sequencer 4000L (LI-COR Inc., Lincoln, NE) were compared, and the clone harboring no artificial point mutation introduced during the PCR process was selected for further analysis.

Fluoroimmunostaining. Cells (synchronized or in log-phase) were cultured on coverslips immersed in a culture dish (ϕ=6cm). They were fixed sequentially by 3.7% formaldehyde, 0.5% Triton X-100 and 0.05% Tween 20 in PBS for 10 min at 20°C. After removing the medium, cells on coverslips were rinsed three times (2ml for 5min each) with TBST buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) supplemented with 2% bovine serum albumin (BSA; Sigma) at room temperature. Coverslips were lifted, and with the cell-side down, placed on 100μl of TBST containing the relevant antibody spotted onto a piece of parafilm. After incubation at room temperature for 1 hr, the coverslips were rinsed with TBST/BSA as described above with the cell-side facing upwards in the culture dishes. FITC- or Texas Red-linked anti-rabbit Ig (from donkey; Amersham) or Texas Red-linked anti-mouse Ig (from sheep; Amersham) was used as a second probe to visualize the immunocomplexes. These second antibodies were incubated with the cells attached to the coverslips for 1 hr and rinsed three times as described above. Photographs were taken and the images were recorded by a inverted laser scan microscope (Zeiss LSM410).

RESULTS AND DISCUSSION

Monospecificity of the Antibodies

For antibody preparations, we synthesized the peptide corresponding to residues No.104-117 of HsMcm7 and used it as an antigen to immunize two different New Zealand White rabbits (#44 and #45). We also synthesized peptides corresponding to residues No.677-700 of HsMcm3 (antibody #27), No.131-153 of HsMcm2 (antibody #60), and No.19-40 of HsMcm5 (antibody #62), and used them as antigens. Since the amino-acid sequence of each of these peptides is only found in the HsMcm protein from which the sequence was derived, we expected that a given antibody would only react with one HsMcm protein. As for HsMcm3, a GST-fusion protein (GST-HsMcm3) was prepared and was used as an antigen (antibody name: MBL). After two months of immunization (third injection of the peptides), rabbit serum was recovered and anti-peptide antibodies was affinity purified by peptide column chromatography, and the specificity of each antibody was examined.

As expected, each antibody was able to recognize its own GST-fusion protein expressed in *E. coli.* after induction with IPTG (Figure 1A). Also, these antibodies associated only with their own GST-Mcm protein without any cross-reactions (data not shown). Antibodies against HsMcm7 (Figure 1B, lanes 4 and 8) and HsMcm3 (Figure 1C, lane 7) were monospecific, recognizing only a single band at the expected size on SDS-PAGE gels. The antibodies against HsMcm2 and HsMcm5 also detected a band of the expected sizes (Figure 1B and Figure 1C, lanes 3 and 10), although they also recognized an additional weaker band. From these results, we considered these antibodies to be of sufficient quality for Western blot analyses.

These antibodies were also useful for immunoprecipitation, since they detected only a single band of the expected size on SDS-PAGE gels that had been loaded with their immunoprecipitates from HeLa cell extracts (Figure 1B, lanes 1, 3, 5 and 7; Figure. 1C, lanes 1, 4, 6 and 8). In these experiments, HeLa cell extracts immunoprecipitated with preimmune serum were used as negative controls.

Mutual Association of HsMcm Proteins

In HeLa cell extracts, only the association of HsMcm4 (12) and HsMcm3 (14) with other members of the Mcm family has been shown previously. To examine whether other HsMcm proteins can also associate with each other in human tissues, we performed recip-

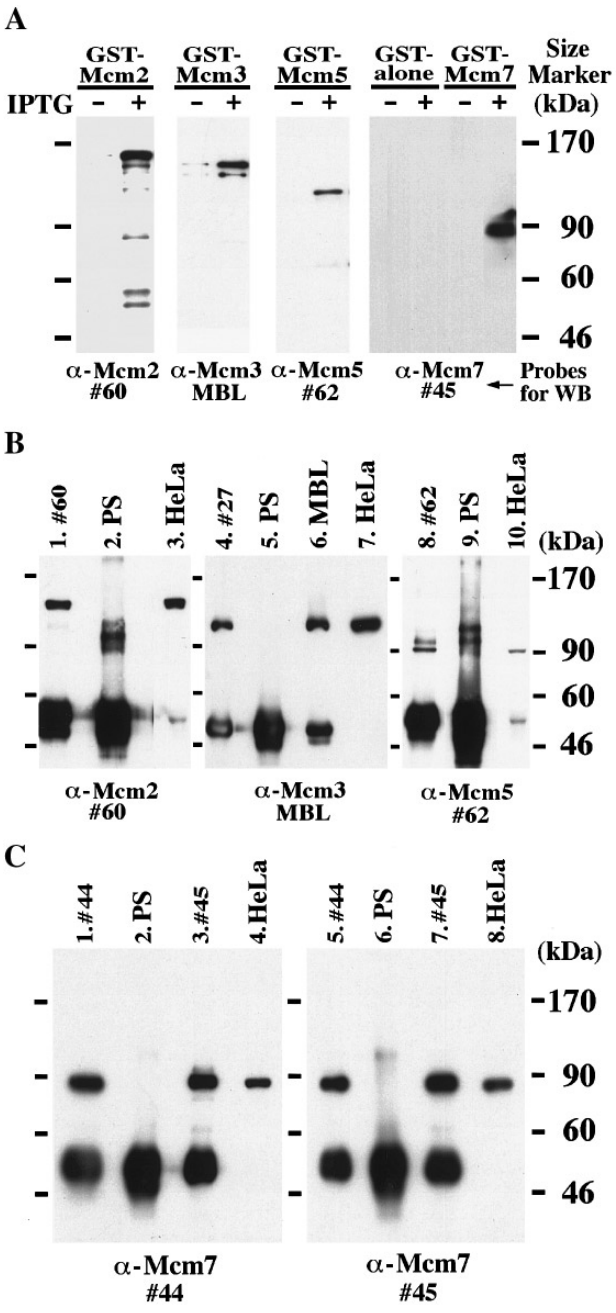

FIG. 1. Characterization of anti-HsMcm antibodies. Anti-HsMcm2, anti-HsMcm3, anti-HsMcm5, and anti-HsMcm7 polyclonal antibodies were obtained by immunizing New Zealand White rabbits with the synthetic peptides, DEEDEERPARKRRQVERATEDGE (HsMcm2), EEEKSQEDQEQKRKRRKTRQPDAK (HsMcm3), AQADEGQARKSQLQRRFKEFLR (HsMcm5) and LEHRLMMEQRSRDPGMVRSPQNQYPAE (HsMcm7), respectively. (**A**) The binding specificity of the affinity purified antibodies to GST fusion proteins of HsMcm2, -3, -5 and -7 expressed in *E. coli* before (−) or after (+) induction with isopropyl-thio-β-D-galactoside (IPTG). To show the absence of a cross reaction between the antibodies and GST or *E. coli* proteins, the GST vector alone was expressed in *E. coli* and the extract was probed with the antibodies. (**B**) Specificity of anti-HsMcm2 (#69), anti-HsMcm3 (#27 and MBL) and anti-HsMcm5 (#62) polyclonal antibodies. (**C**) Specificity of the anti-HsMcm7 polyclonal antibodies (#44 and #45). Immunoprecipitation and Western blot (WB) analyses were performed using the HeLa cell extract (500μg). An immunoprecipitate obtained with preimmune serum (PS) was loaded onto the SDS-PAGE as a negative control. HeLa cell extract (20μg) was also electrophoresed to assign the bands for HsMcm proteins.

rocal immunoprecipitation and western blot analyses using antibodies against HsMcm2, -3, -5 and -7. As shown in Figure 2B, immunoprecipitates obtained using anti-HsMcm2, -3 or -5 antibodies (lanes 1-3), but not preimmune serum (lane 5), displayed a band at 85 kDa that migrated to the same position as a band immunoprecipitated with anti-HsMcm7 antibodies (lane 4), suggesting an association of HsMcm7 with HsMcm2, -3 and -5. Furthermore, this band could be resolved into doublet bands at lower exposures of the film (lane 7), and it appeared that HsMcm2, -3 and -5 preferentially associated with the HsMcm7 species represented by the lower band.

The possibility existed that the lower band represents a phosphorylated form of the protein, since HsMcm7 contains ten potential phosphorylation sites for protein kinase C or casein kinase II (Figure 2A), and one of the recognition sites, TVRE at residue number 664, is recognized by both of these protein kinases. To examine this, we radiolabeled the protein by incubating HeLa cells in the presence of ^{32}P-orthophosphate and immunoprecipitated the cell extracts with anti-HsMcm7 antibody. The proteins in the immunoprecipitates were then separated on SDS-PAGE, transferred to a nitrocellulose filter and exposed to an X-ray film. We detected two phosphorylated bands around 85 kDa (Figure 2C, lane 1), which were not present when the extract was immunprecipitated by preimmune serum (lane 2). Next, we showed that these two bands correspond to Hsmcm7 by using the same filter for Western analysis with anti-HsMcm7 antibody (lane 3). The results indicate that both of these two immunprecipitated bands are phosphorylated forms of HsMcm7.

As shown in Figure 2D, immunoprecipitates obtained using anti-Mcm7 antibody, but not preimmune serum (lanes 3, 7 and 11), also displayed bands corresponding in size to HsMcm2 (lanes 1, 2 and 4), HsMcm3 (lanes 5, 6 and 8) and HsMcm5 (lanes 9, 10 and 12), when probed by the relevant antibodies. Thus, the results obtained here indicate that HsMcm2, -3, -5, and -7 are components of the large Mcm complex.

All HsMCM/P1 mRNA Levels Peak at the Late G1 to S Phase

If a large amount of Mcm/P1 is required for the initiation of S phase, the expression of these genes would be expected to increase as cells approach S phase. To examine this, we performed Northern analysis of NRK (normal rat kidney) cells in a growth arrested cell population ob-

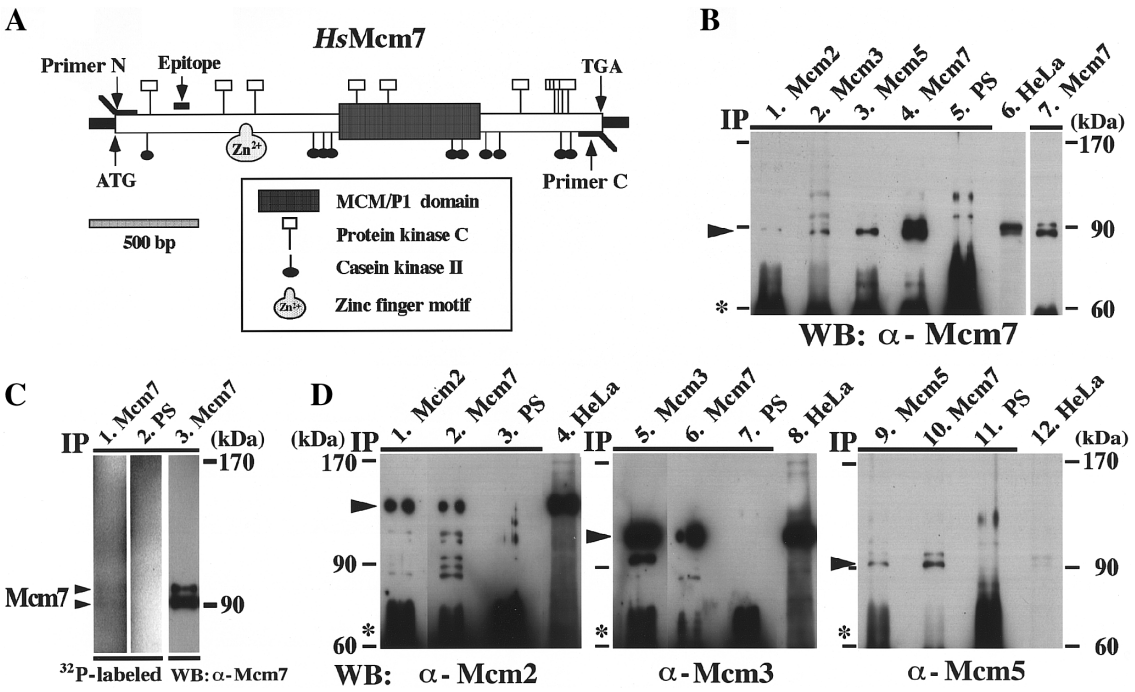

FIG. 2. HsMcm2, -3, -5, and -7 are present as a multiprotein complex. (**A**) A schematic presentation of the structure of the cDNA encoding HsMcm7. The location of the amino acids synthesized to raise the polyclonal antibody is denoted as **Epitope.** The accession number of *HsMCM7* in the DDBJ/EMBL/GenBank is D86748. (**B**) HeLa cell extracts were immunoprecipitated (IP) with anti-HsMcm2, -3, -5, -7 or preimmune serum (PS) and the precipitates were subjected to Western blot (WB) analysis using the anti-HsMcm7 antibody as a probe. HeLa cell extract (20μg) was also electrophoresed to assign the band for HsMcm7 (lane 6). An image of lower exposure of lane 4 is presented (lane 7) to allow resolution of the doublet bands. (**C**) HsMcm7 is a phosphoprotein. HeLa cells were ^{32}P-labeled *in vivo,* and immunoprecipitated (IP) by anti-HsMcm7 antibody or preimmune serum (PS). Arrowheads denote the bands for HsMcm7. (**D**) Reciprocal immunoprecipitation and Western blot analyses of the immunoprecipitates of anti-HsMcm7 antibody as probed by anti-HsMcm2, -3 or -5 antibodies. Arrowheads denote the bands for HsMcm2, -3 or -5. Asterisks indicate the band for the heavy chain of immunoglobulin.

tained by serum depletion or in a growing population obtained after serum replenishment. A synchronized transition from G0 to the G1/S phase may be observed by following the time course after serum replenishment. NRK cells were starved by serum depletion for 2 days and the cell cycle was initiated by the addition of serum to the cell cultures. FACS analysis indicated that successful synchronization had been achieved (data not shown). The percentage of the cell population in G1, S or G2/M phases as measured by the FACS patterns is listed above the Northern blots. As shown in Figure 3A, the mRNA levels of the *HsMCM2-7* genes were low, although they were detectable during the quiescent state (G0 phase). After addition of serum, however, *HsMCM2-7* gene transcription increased abruptly at 9 hours, when the cells were in late G1 phase. The maximum level was maintained for 9 to 15 hrs, and then suddenly decreased as the cells approached a second round of G1 phase (at 24 hours). In this experiment, the almost homogeneous intensity of the GAPDH mRNA served as a loading control. From these results, we conclude that all of the *MCM/P1* genes responded almost identically to growth stimulation before the initiation of DNA synthesis, suggesting a crucial requirement of these proteins for the start of S phase.

We next examined the structure of the 5′ upstream region of one of these genes. For this purpose, we cloned the 5′ regulatory region of the *HsMCM5* gene by PCR using the promoter finder technique (Stratagene, La Jola, CA), since it alone showed almost no transcription during G0 phase (0 hr). DNA sequencing revealed the existence in this region of a variety of sequence motifs that could serve as potential binding sites for transcriptional regulatory factors (Figure 3B). Among these, two E2F motifs, situated at -194 and -183, are possible candidates for the regulation of G1/S expression of the *HsMCM5* gene, as the E2F motif has been shown to regulate a group of genes that are commonly expressed in late G1 and which play pivotal roles in DNA replication. Another notable motif is the adenine rich sequence (underlined in Figure 3B), which was also found in the 5′ upstream region of the mouse MCM3 gene (10).

Expression of HsMcm Proteins during the Synchronized Cell Cycle

Next, we wished to investigate whether cell cycle dependent changes in mRNA levels result in fluctuations

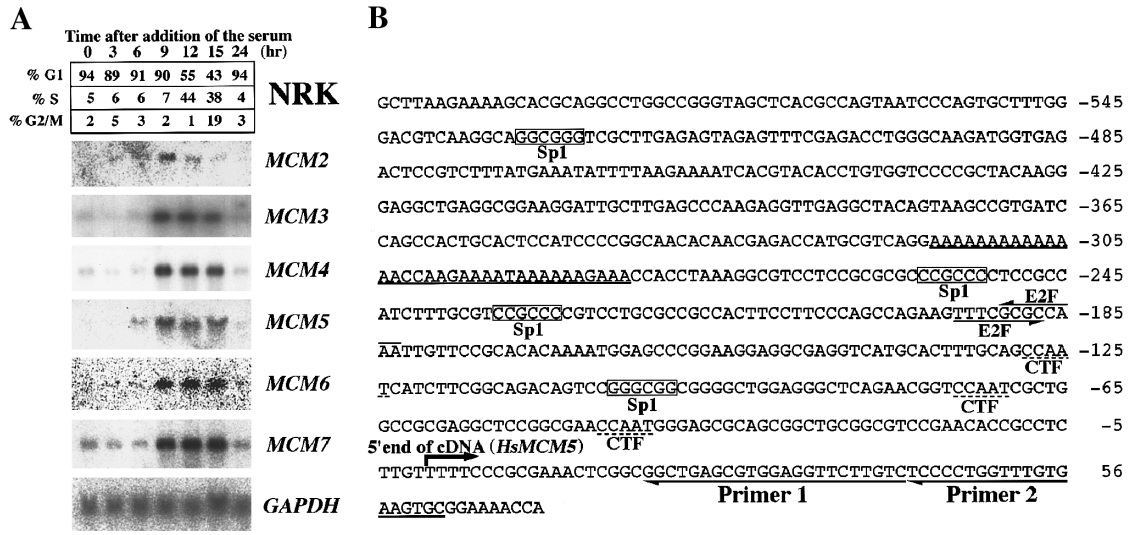

FIG. 3. Northern analyses and the structure of the 5′ upstream of *HsMCM5*. (**A**) The mRNA level of *MCM/P1* genes as assessed by Northern blot analyses during the cell cycle of NRK cultures synchronized by serum starvation. The mRNA level of the GAPDH (glyceraldehydephosphate dehydrogenase) gene is shown below as a loading control. The percentages of cell populations in G1, S or G2/M phases for each sample, as calculated by FACS analysis, are shown above. (**B**) The DNA sequence of the 5′ flanking region of the *HsMCM5* gene with the Sp1, CTF and E2F motifs indicated. Nucleotide numbering starts at the 5′ end of the cDNA. The locations of the two primers used for PCR are indicated by arrows. The accession number in the DDBJ/EMBL/GenBank is AB003469.

in HsMcm protein levels. To address this question, we directly compared the oscillations in the protein levels of the four kinds of HsMcm protein during the synchronized cell cycle by Western blot analysis. Identical amounts of whole cell extract were loaded into each lane of an SDS-PAGE gel, transferred to a nitrocellulose filter and probed with HsMcm2, -3, -5 and -7 antibodies. In order to correct for any differences in the amounts of cell extract loaded in each lane, one of the membranes was probed by an antibody against anti-tubulin, whose concentration is known not to vary throughout the cell cycle. We tested two distinct types of cell cycle progression using normal cells (KD) and cancer cells (HeLa).

First, we examined a growth arrested cell population of KD (human lip fibroblast; primary culture) cells ob-

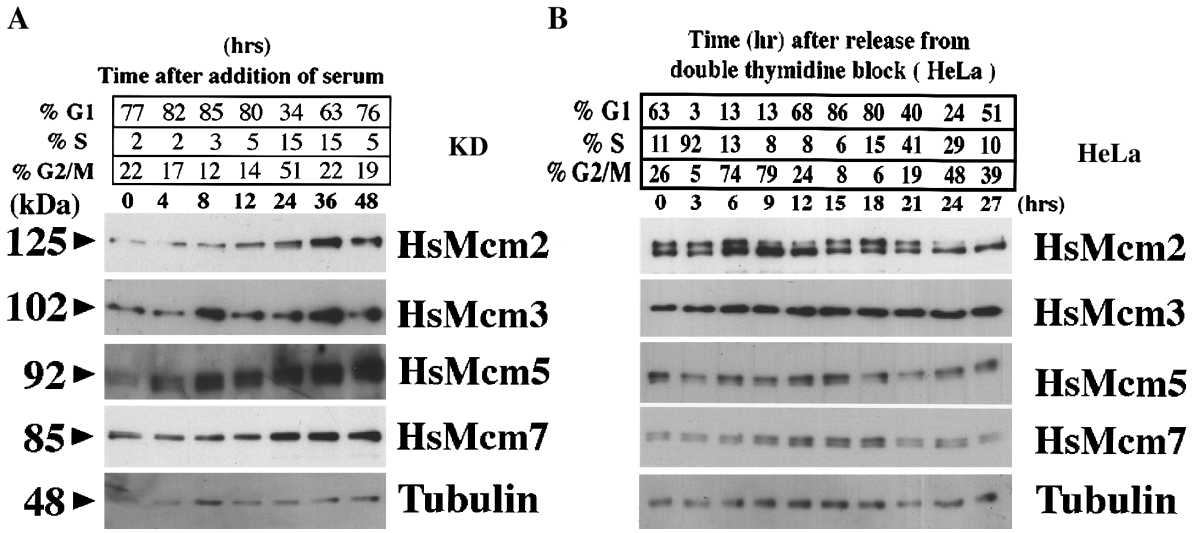

FIG. 4. Expression of HsMcm proteins during the synchronized KD or HeLa cell cycle. (**A**) Western blot analyses of total cell extracts (10 μg) from KD cultures synchronized by serum depletion. (**B**) Western blot analyses of the total cell extract (10 μg) of HeLa cells synchronized by double thymidine block. Anti-HsMcm2, -3, -5 and -7 polyclonal antibodies and anti-tubulin monoclonal antibody were used as probes. Percentages of cell populations in G1, S or G2/M phases, calculated by measuring the areas of the peaks in the FACS analysis, are shown above the blots.

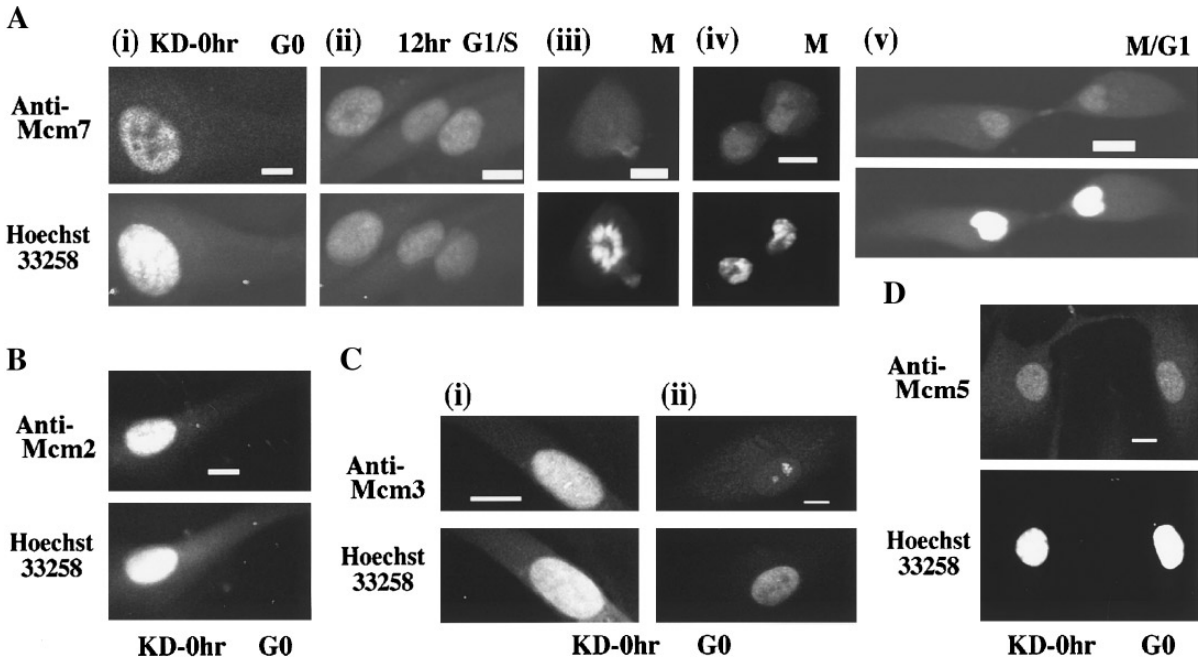

FIG. 5. Nuclear localization of HsMcm proteins at G0 phase. KD cells grown on coverslips were growth-arrested by serum depletion (0 hr). Then, the cell cycle was initiated by the addition of the serum. Cells were fixed at the indicated time points with formaldehyde, permeabilized with Triton X-100 and Tween 20, and stained with the affinity purified anti-HsMcm7 (**A**), anti-HsMcm2 (**B**), anti-HsMcm3 (**C**) or anti-HsMcm5 (**D**) antibodies. The localizations of the bound antibodies were visualized by Texas red-linked anti-rabbit IgG antibody under a confocal microscope (Zeiss LSM410). The localization of the nuclei was also determined by staining the cells with Hoechst 33258. As for HsMcm7, the images corresponding to the quiescent state (G0) (i), G1/S phase (ii) or M phases (iii-v) are sequentially arranged. As for HsMcm3, two typical images at the G0 phase are shown (panels i and ii).

tained by serum depletion or a growing population of these cells obtained after serum replenishment. A synchronized transition from G0 to the G1/S phase in all the cells is expected to occur after the addition of serum to serum-starved cells. Thus, we starved KD cells by serum depletion for 2 days, and subsequently replenished the medium with serum to initiate the cell cycle. We performed FACS analysis in order to confirm successful synchronization, and the percentage of the cell population in G1, S or G2/M phase was measured using FACS analysis, the results of which are displayed above the Western blots. As shown in Figure 4A, the protein levels of these HsMcm proteins gradually increased after release of the cell population from the G0 state and as the cells progressed into the G1/S phase. This is consistent with the result shown in Figure 3A, and our previous result showing that the mRNA levels of serum starved KD cells also increase after serum replenishment at the G1/S transition (16). Of particular interest is the fact that the oscillation profile of each HsMcm protein was distinct. The intensity of the bands for HsMcm2, -3, -5 or -7 at 36 hrs were respectively 3.3, 2.9, 4.8, 1.6 times more intense compared to those seen at 0 hr (analyzed by Densitograph, ATTO Co., Ltd, Tokyo). The result indicates that the amounts of these HsMcm proteins are not stoichiometric during the G1/S transition.

Secondly, we examined a cancer cell line (HeLa), whose cell population cannot enter into the G0 state because of anchorage independent growth. In this case, cell cycle synchronization was attained by a double thymidine block, which allowed the cells to begin the cell cycle at S phase and to sequentially progress into the G2/M phase and G1/S transition after removal of the thymidine block. A portion of the cell population was collected every 3 hours, and subjected to FACS and Western blot analyses. Successful synchronization was again confirmed by FACS analysis. As shown in Figure 4B, oscillations in the protein levels of HsMcm2, -3, -5 and -7 were minor and the profiles were similar to that of tubulin, indicating the maintenance of constant levels of these proteins throughout the period following initiation of the cell cycle. This is in contrast to our previous report showing that the mRNA levels of these *HsMCM* genes fluctuate during this period and peak during the G1/S phase (16). The result suggests that the regulatory mechanisms governing the levels of these HsMcm proteins during cell cycle progression are distinct from those of the G0 state. Another notable point was that HsMcm5 and -7 displayed doublet bands but the ratio of their intensities did not change throughout the cell cycle. On the other hand, the upper band of the HsMcm2 doublet, which is known to be the underphosphorylated form (17), showed large oscilla-

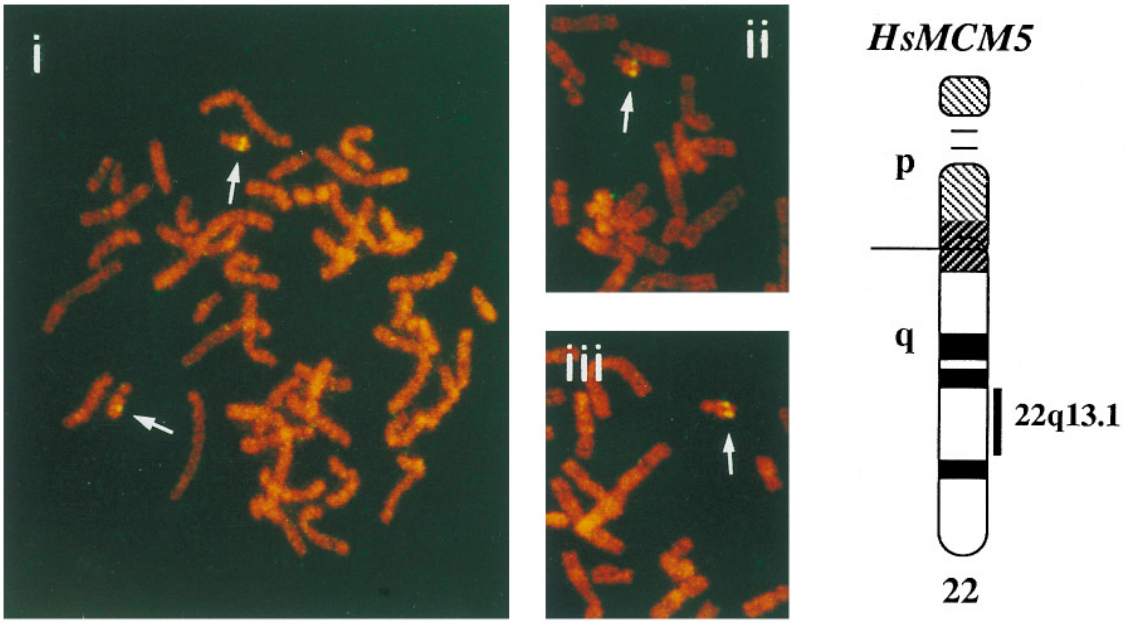

FIG. 6. FISH using cDNA probes of the *HsMCM5* gene on R-banded human chromosomes. The entire metaphase (**i**) and partial metaphases (**ii and iii**) are shown. Arrows indicate specific FITC signals for the *HsMCM5* gene using biotin-labeled DNA fragments of relevant cDNA inserts as probes. Separate images of FITC-hybridization signals and PI-stained chromosomes were merged using image analysis software, and photographs were taken from digital images without computer-assisted modification through a camera attached to a Zeiss LSM. A schematic presentation of chromosomes 22, and the location of the gene is shown on the right panel.

tions with a peak at the G1/S transition. Such doublet bands were not observed during the G0 to G1/S period for HsMcm2 and -7. The cause of the difference in the phosphorylation patterns revealed by these two experimental systems remains to be clarified.

From these results, we conclude that the amounts of HsMcm proteins remain constant during the cell cycle except for variations during the G0 to G1/S period. Our results also indicate that these Mcm proteins are not present in equivalent amounts. This is especially the case during the quiescent state (G0), where the amounts of HsMcm2 and -5 proteins were much lower than those of HsMcm7 and -3. These results suggest that a certain proportion of these molecules may exist in a free form, located away from the Mcm/P1 complex.

Nuclear Localization of HsMcm2, -3, -5 and -7 Proteins during the Quiescent State

Although the subcellular localization of human HsMcm/P1 proteins has been studied in rapidly growing HeLa cells (13, 17, 18), their specific localization during the G0 and M/G1 phases has not been clarified. Therefore, we investigated this using our antibodies. We prepared synchronized KD cells on coverslips at the same time as we prepared cell extracts for Western blot analysis as described above (Figure 4A). For comparison, DNA in the nucleus was microscopically visualized under UV light after Hoechst 33258 staining. As shown in Figure 5, quiescent cells at the G0 phase displayed intense staining for these HsMcm proteins in their nuclei. It is noteworthy that only in the case of HsMcm3 did 50% of the cell population display a granulated distribution at a restricted location in the nucleus, which probably corresponds to the nucleolus (Figure 5C-ii). This unusual localization in the nucleus during the G0 phase was not observed for other HsMcm proteins (Figure 5A-i, B and D). During the G1/S phase, all of these proteins were observed in the nucleus (Figure 5A-ii; data not shown for other HsMcm proteins to save space). During pro- and metaphase, most of these HsMcm proteins became excluded from the chromatin (panel iii) at the time of nuclear membrane breakdown. During anaphase (panel iv), condensed chromatin was still free from these HsMcm proteins. At telophase (panel v), the HsMcm proteins re-entered the nucleus and reassociated with the decondensed chromatin. Here, the timing of reassociation of these HsMcm proteins with chromatin appeared to happen before that of nuclear formation. Taken together, our results indicate that the behavior of these HsMcm protein during the cell cycle is similar to the behavior of the hypothetical replication licensing factor.

Chromosomal Location of HsMCM5 and HsMCM7 Genes

We mapped the chromosomal location of the *HsMCM5* and *HsMCM7* genes by the fluorescence *in*

situ hybridization (FISH) technique as described before (19). Specific FISH signals generated from biotin-labeled cDNAs of *HsMCM5* and *HsMCM7* were observed on the short arm of chromosome 22 (22q13.1) for *HsMCM5* and on the short arm of chromosome 7 (7q21) for *HsMCM7* in the majority of the metaphase chromosomes examined (Figure 6; data not shown for *HsMCM7*). Nakatsuru *et al.* (20) mapped a gene, which they called human Mcm2, on chromosome 7 (7q21), which is the same location as our *HsMCM7* gene. By checking their cDNA sequence, however, it is apparent that they chose an inappropriate name for their Mcm protein in naming it Mcm2, when it should have been referred to as the *HsMCM7* gene. These sites are distinct from those of *HsMCM2* at 3q21 (21), *HsMCM3* at 6p12 (22), and *HsMCM6* at 2q12-14 (16), indicating that *HsMCM* genes are not clustered on the genome.

In summary, the results presented here indicate that these four HsMcm proteins undergo unequal regulation, suggesting that they play somewhat distinct roles in the regulation of the mammalian cell cycle.

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