Site-specific Loading of an MCM Protein Complex in a DNA Replication Initiation Zone Upstream of the c-MYC Gene in the HeLa Cell Cycle

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The MCM proteins participate in an orderly association, beginning with the origin recognition complex, that culminates in the initiation of chromosomal DNA replication. Among these, MCM proteins 4, 6, and 7 constitute a subcomplex that reportedly possesses DNA helicase activity. Little is known about DNA sequences initially bound by these MCM proteins or about their cell cycle distribution in the chromatin. We have determined the locations of certain MCM and associated protein complexes in chromatin immunoprecipitates (ChIP) from the c-MYC gene in the HeLa cell cycle. MCM7 and its clamp-loading partner Cdc6 are highly specifically colocalized by ChIP and re-ChIP in G1 and early S on a 198-bp segment located near the center of the initiation zone. ChIP and Re-ChIP colocalizes MCM7 and ORC1 to the same segment specifically in late G1. MCM proteins 6 and 7 can be coimmunoprecipitated throughout the cell cycle, whereas MCM4 is reduced in the complex in late S and G2, reappearing upon mitosis. MCM7 is not visualized by immunohistochemistry on metaphase chromosomes. MCM7 is recruited to multiple sites in chromatin in S and G2, at which time it is not detected with ORC1. The rate of dissemination is surprisingly slow and is unlikely to be simply attributed to progression with replication forks. Results indicate sequence-specific loading of MCM proteins onto DNA in late G1 followed by a recruitment to multiple sites in chromatin subsequent to replication.

Members of the minichromosome maintenance (MCM)† protein family were first characterized in Saccharomyces cerevisiae as essential for plasmid maintenance during the cell cycle (reviewed in Refs. 1–3). Each of the six proteins MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 has a counterpart in yeast, Schizosaccharomyces pombe (reviewed in Refs. 1–3). Each of the six proteins MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 has a counterpart in yeast, Schizosaccharomyces pombe (reviewed in Refs. 1–3). The helicase activity of an MCM-like protein from archaeabacteria (22). The helicase activity of an MCM4,6,7 complex, reconstituted from recombinant S. pombe proteins, reportedly requires the presence of forked DNA structures (18). Although results taken together suggest that the MCM4,6,7 complex could represent a major replicative helicase, there is little direct evidence for this. In particular, it is not known whether this complex selectively assembles at initiation zones of DNA replication in mammalian cells. Recent reports have dealt with cell cycle distribution of MCM proteins in chromatin in the hamster DHFR locus (23, 24), but these did not apparently mark "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: MCM, minichromosome maintenance; pre-RC, pre-replication complex; ORC, origin recognition complex; FACTS, fluorescence-activated cell sorting; ChIP chromatosomal immunoprecipitation; re-ChIP, re-precipitation; PBS, phosphate-buffered saline; DAB, diaminobenzidine tetrahydrochloride; nt, nucleotide(s); GFP, green fluorescent protein.

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EXPERIMENTAL PROCEDURES

Cell Culture and Cell Cycle Synchrony—HeLa cells were maintained in spinner culture in Eagle's Minimal Essential Medium for suspension cultures (with α-glutamine and without calcium and magnesium, Cellgro Mediatech) supplemented with 10% (v/v) fetal bovine serum (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO₂, 95% air. Cell cycle synchrony was achieved using a double thymidine block, exactly as described previously (34). By this method cells are blocked at the beginning of S-phase. Cell cycle progression was verified by FACS analysis (Fig. 1A), and timing of mitosis was verified by microscopic determination of the mitotic index. To specifically examine time points at mitosis the double thymidine block was employed in conjunction with the microtubule disruptor, nocodazole. Eight hours after release from the double thymidine block, the HeLa cells were treated with nocodazole (methylethylimidazole-2-yl) carbamate, 100 ng/ml, Sigma-Aldrich) for 4 h. This treatment blocks the already synchronized cells at the G2-M boundary (35). At that time the cells were released from nocodazole into new medium, and time points were taken for analysis at 1-h intervals.

Chromatin Immunoprecipitation (ChIP) and Re-precipitation (re-ChIP) to Detect Proteins Bound to DNA Sequences Upstream of the c-MYC Gene—Approximately 2 × 10⁷ HeLa cells were collected every 2 or 2.5 h after release from double thymidine block (34). Chromatin immunoprecipitation was done using a modification of the procedure of Kuo and Allis (36). Cells were incubated for 15 min in medium containing 1% formaldehyde at room temperature, and cross-linking was quenched by adding glycine to 125 mM. Cells were washed with ice-cold Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.6) three times. Cell pellets were resuspended in 500 μl of lysis buffer (0.1% deoxycholic acid, 1 mM EDTA, 50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 50 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml PMSF), and cells were disrupted on ice with 20 strokes of a Dounce homogenizer. Sonication was performed by pulsing three times for 15 s, incubating on ice for 2 min between each pulse, followed by centrifugation at 15,000 × g for 5 min to remove cell debris. Gel electrophoresis indicates that a substantial fraction of DNA fragments at this stage are from 0.3 to 3.0 kb in length. Centrifugation was performed for another 15 min to obtain cleared cell lysate. ChIP was performed on the cell lysate by overnight incubation at 4 °C with 4 μg of primary antibody followed by incubation with protein A-Plus-agarose (Santa Cruz Biotechnology) for 2 h. The beads were rinsed four times sequentially with lysis buffer 500 (0.1% deoxycholic acid, 1 mM EDTA, 50 mM HEPES, pH 7.5, 500 mM NaCl, 1% Triton X-100), LiCl/detergent solution (0.5% deoxycholic acid, 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 8.0), and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The beads were then incubated for 10 min at 65 °C with elution buffer (10 mM Tris-EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.0) to elute the precipitates. For re-ChIP, re-ChIP elution buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.7 μl NaCl) was used to elute. The elution buffer was diluted to 1/5, and re-ChIP was performed using a second primary antibody as for the first ChIP. To reverse the cross-linking and purify the DNA, precipitations were incubated in a 65 °C incubator overnight and then incubated with protease K solution (40 μg/ml glycogen and 0.4 mg/ml protease K stock containing 50 mM Tris-HCl, pH 8.0, and 1 mM CaCl₂ in TE buffer, pH 7.6) for 2 h at 37 °C. DNA samples were then purified using LiCl and phenol/chloroform/isoamyl alcohol. DNA was precipitated by adding ethanol to 70%, and precipitates were washed with 75% ethanol, air-dried, and resuspended in TE buffer.

PCR Amplification of DNA Sequences Obtained by ChIP—The following primers were employed to amplify genomic sequences upstream of the human c-MYC gene. Locations of the primers by primer sequences from the HindIII site at 2325 nt upstream of exon I specified by Pf promotor. myc5F 198, 5′-AACCGATATTTGCGATGTCAT-3′ (4845–4866, 22-mer); myc3R segment c, 5′-CCCTCACCAACGTGAAT-3′ (725–705, 21-mer); myc5F 350, 5′-CTCTCCTTTTTCAGACTACG-3′ (1052–1072, 21-mer); myc3R segment b, 5′-CTCAATTTCTACGACCTGTTT-C-3′ (1401–1381, 21-mer); myc5F segment a, 5′-CTGGCTGGAATACCTC- TTTC-3′ (175–185, 20-mer); myc3R segment a, 5′-CTCTACTGCGGCACCA- GCAGAGATC-3′ (373–354, 20-mer); myc5F segment b, 5′-CAGGGTT- AACCGAGGTTCACCC-3′ (4845–4866, 22-mer); myc3R segment b, 5′- CTCGATTCGTCAGAAC-3′ (5063–5043, 21-mer); myc5F segment c, 5′- CAAAGGACCGACATGCAGAGAC-3′ (379–3780, 22-mer), myc3R segment c, 5′-CATACCTGACCGTGGCCTTC-3′ (3598–3578, 22-mer); myc5F segment d, 5′-GGTGTCCACACCTGATGTAGT-3′ (62426–62447, 22-mer); myc3R segment d, 5′-GAGAATCTCTGCTCTG- CCAG-3′ (62805–62825, 21-mer); myc3R segment e, 5′-GAAATCTCTGCTGTGCCCAC-3′ (63151–63131, 21-mer); and myc3R segment e, 5′-GTCCTCTTATGTATGCAAC-3′ (61170–61149, 22-mer).

PCR reactions were performed as previously described (37). Reactions were carried out with 0.2 μM of each dNTP, 0.3 μM primer/reach, 10 × PCR buffer with Mg²⁺, and 1.75 unit of enzyme mix of the High Fidelity PCR System (Roche Applied Science). Hot start PCR reactions were carried out for either 34 or 42 cycles as indicated. PCR reactions were analyzed by electrophoresis on 2% agarose gels stained with SYBR Gold nucleic acid gel stain (Molecular Probes).

Immunoblotting, Immunoprecipitation, and Detection of Proteins—Total HeLa cell lysates were produced by Dounce homogenization in a lysis buffer (150 mM NaCl, 0.1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, in PBS (137 mM NaCl, 2.7 mM potassium chloride, 4.2 mM sodium phosphate, and 1.5 mM potassium phosphate)). Cell lysates were cleansed by centrifugation. SDS-PAGE and immunoblot transfers of proteins to Immobilon-P membranes were performed as described previously (38, 39). The Supersignal chemiluminescence system (Pierce) was used for detection. Antibodies for MCM2 (N-19 goat polyclonal), MCM3 (N-19 goat polyclonal), MCM4 (K-18 goat polyclonal), MCM6 (C-20 goat polyclonal), MCM7 (141.2 mouse monoclonal), ORC1, in a well characterized initiation complex, their clamp-loading partner Cdc6, and their partner of the human c-MYC locus, MYC

RESULTS

Timing of Specific Localization of MCM7 in c-MYC Chromatin During the HeLa Cell Cycle—We sought a method by which we could compare simultaneous localization of a given protein at multiple points over an extended genomic sequence region. Real-time PCR was not considered practical here, because, although it provides excellent quantitation of sequence representation in a chromatin sample obtained by ChIP, the method becomes cumbersome when applied to multiple sequence locations in multiple
cell cycle time points. The high degree of synchrony achieved by the double thymidine block method is shown in Fig. 1A. The adaptation of chromatin immunoprecipitation (ChIP) detailed in Fig. 1B allows assessment of the presence of a given MCM protein at two locations upstream of the c-MYC gene and, simultaneously, on an extended region between the two locations throughout the cell cycle. Fig. 1C shows the positions of two primer sets used to amplify the three indicated PCR segments. The 198-bp segment is near the center of the replication initiation zone originally mapped upstream of c-MYC (29). It contains a prominent site of DNA bending and a purine-rich recognition element for the single-stranded DNA-binding protein, PurA (40, ...
ChIP of DNA Segments at Different Distances from the Initiation Zone Reveal a Dissemination of MCM7 throughout S- and G2-phases—If the presence of MCM7 is expanding outward from an initial assembly point during ongoing replication, then PCR segments that are the same size as the initial segment, but distally located, should yield results similar to those of the 350- and 876-bp segments when analyzed over the cell cycle, and the appearance of MCM7 in the more proximal segments should occur earlier than in the more distal segments. Fig. 2 shows that there is a dissemination of MCM7 to distal chromatin sites, but that dissemination is not a simple progression from a central point. Five segments have been used (a–e), all of which are at different sites from the segments used for Fig. 1. The positions of these segments are indicated in the two maps at the bottom of Fig. 2. It appears that there is increasing distribution to the segments a–d during S and G2, similar to those seen with the 350- and 876-bp segments of Fig. 1. MCM7 appears, however, in segments b and d earlier than it does in segment a, which is much closer to the 198-bp site of initial appearance. The segments closest to the initiation zone center, a–c, show the sharpest peaks of MCM7 location, from 6 to 10 h. Segments b and c are each about 4 kb from the 198-bp segment of Fig. 1, and their patterns are quite similar to that of the 350-bp segment of Fig. 1. This similar peaking may be anticipated if there is outward progression from a central segment, but the timing of MCM7 appearance is much slower than expected. Mammalian replication forks are believed to progress at >100 nts/zoom. Aspects of the rates of distribution of MCM7 to these segments will be considered in the discussion. Segment e shows a pattern similar to that of the 198-bp segment in Fig. 1. This segment is nearly 60 kb away from the center of the 198-bp segment and may be in or near another replicon. The patterns observed with segments a–d indicate that there is dissemination of MCM7 to multiple chromatin sites during DNA replication. If this dissemination involves progression, however, that must be coupled with an alternate, and slower, form of recruitment.

Double ChIP Reveals That MCM7 and Cdc6 Are Present Together on the 198-bp c-MYC DNA Segment Specifically at Cell Cycle Times of Initiation of Replication—The notion that MCM7 is specifically localized on DNA at initiation and is then distributed during ensuing replication was further examined in an experiment employing ChIP and re-ChIP. Cdc6 has been implicated in the loading of MCM proteins onto DNA to form the pre-initiation complex (5, 11). We hypothesized that if Cdc6 is involved in loading MCM7 onto chromatin, then these two proteins would be specifically localized together at times of initiation. ChIP and re-ChIP offer an ideal means to test this. First ChIP was performed using anti-Cdc6 antibody. Then, prior to reversal of protein-DNA cross-linking, the chromatin fragments were subjected to re-precipitation using anti-MCM7 antibody. During subsequent PCR only those DNA sequences would be amplified that are simultaneously bound to both proteins. It can be seen in Fig. 3A that Cdc6 and MCM7 are highly specifically colocalized to the same DNA sequences in the 198-bp c-MYC segment at early S-phase (2.5 h) and mid-to-late G1-phase (17.5 h). Subsequently, throughout S and G2, these proteins are distributed to sequences in the 350-bp segment. Distribution to the 876-bp segment (not shown) essentially parallels that to the 350-bp segment. Although Cdc6 and MCM7 can be detected together on DNA at all cell cycle points tested, their highly specific colocalization to the 198-bp band occurs before and just after the onset of S-phase.

A number of controls are required to address questions arising from the data presented. For example, is it possible that sequences representing one of the segments amplified are over-
represented in the sonicated chromatin starting material and are thus preferentially immunoprecipitated? Furthermore, is it possible that, in the multiple-segment PCR reaction performed, one of the segments is preferentially amplified over the others depending on the amount of template DNA present? Controls in Fig. 3 address these questions. The left panel of Fig. 3 shows PCR bands from a sample representing primarily G1-phase. It can be seen that in chromatin immunoprecipitated by Cdc6 alone (IP Cdc6), sequences of the 350-bp segment are highly represented. After re-ChIP the fraction unbound by the anti-MCM7 antibody (ub MCM7) still contains a high representation of these 350-bp segment sequences. In contrast, the fraction immunoprecipitated by anti-MCM7 (re-IP MCM7) is highly enriched in the 198-bp segment sequences. Thus, the specific immunoprecipitation of the 198-bp segment sequences is not simply due to their excess representation in the total chromatin starting material. The right panel of Fig. 3B presents a concentration course of input template DNA over a dilution range from $10^{-1}$ through $10^{-6}$-fold. It can be seen that at no concentration is the amplification of the 198-bp band preferential. Therefore, the specific detection of the 198-bp segment sequences in immunoprecipitated chromatin is not due artifactually to its preferential amplification.

Following the observation that Cdc6 and MCM7 are highly specifically colocalized on DNA in G1 and early S, and that MCM7 is itself specifically localized, we proceeded to examine Cdc6 alone. Previous studies have indicated that levels of Cdc6 are particularly low in G1 of the HeLa cell cycle (17). We also found that to be true (Fig. 4, 2.5- and 15-h lanes), an intriguing result given that Cdc6 and MCM7 are maximally colocalized...
ChIP was performed using anti-Cdc6 antibody as described under "Experimental Procedures." Prior to reversal of formaldehyde cross-linking, precipitates were washed, resuspended, and subjected to re-ChIP using anti-MCM7 antibody as described. PCR was then performed as described to analyze presence of three segments upstream of the c-MYC gene using anti-MCM7 antibody. Re-IP MCM7 fraction shows considerable specificity for the 198-bp segment, as was previously documented in Fig. 1A.

**Cell Cycle Disposition of an MCM4,6,7 Complex**—Fig. 5A shows that anti-MCM7 coimmunoprecipitates MCM4, and Fig. 5B shows that anti-MCM4 and anti-MCM6 each coimmunoprecipitates MCM7. If these proteins are complexed after synthesis, then one might expect them to be loaded onto chromatin together. As S-phase commences, then the MCM4,6,7 complex becomes loaded onto chromatin in G1-phase. From 12.5 to 15 h Cdc6 alone is at a low level in the 350-bp segment, while at these times MCM7 alone (Fig. 1B) and MCM7 together with Cdc6 (Fig. 3A) are relatively high on that segment. One may ask why the double-ChIP experiment of Fig. 3 looks so different from the single ChIP experiments of Figs. 1 and 4. The answer is that the double-ChIP actually detects the simultaneous presence of two proteins on a single segment of DNA. A band may appear intense when ChIP is done with either MCM7 (Fig. 1) or with Cdc6 (Fig. 4), but the two proteins may not be on that segment simultaneously. Despite the wide distribution of Cdc6 in chromatin over much of the cell cycle, its copresence with MCM7 at the same DNA site is an event triggered with great precision in G1 and early S.

**Presence of MCM4 in the MCM4,6,7 Complex Is Diminished in S and G2 and Re-established during Cell Division**—Nocodazole synchrony was employed to examine the nature of the MCM4,6,7 complex during mitosis. For these studies HeLa cells were synchronized by double thymidine block as usual and allowed to progress to G2 (8 h), at which time nocodazole was added to the medium. This microtubule disruptor blocks the cells prior to chromosome condensation and alignment of the metaphase plate. Upon removal of nocodazole from the medium, at 12 h, microtubule assembly is re-established, and mitosis can commence. The graph at the bottom of Fig. 6 shows that cell division takes place from 2 to 4 h after release from nocodazole. Immunoprecipitation with anti-MCM6 was used to coimmunoprecipitate MCM proteins 4 and 7 from extracts of asynchronous cells and from cells at all time points shown in the graph. It can be seen that MCM6 and MCM7 remain constantly in a complex at all times. As S-phase commences upon release from the double thymidine block, MCM4 is diminished in the complex. As cell division proceeds, MCM4 levels increase in the complex. If the MCM protein complex is loaded onto specific sites of DNA in late G1, then one might expect them to be loaded onto chromatin as suggested by the double-ChIP experiment of Fig. 3A, then the MCM4,6,7 complex is formed in cells prior to its actual loading.

**MCM7 Is Not Visualized on Chromatin During Metaphase in Cervical and Ovarian Cancer Tissues**—Mitosis, which occurs at ~11.5 h after release from the double thymidine block, is not adequately dealt with in the data of Figs. 1B and 3A. Chromosome condensation and separation occur very rapidly in the
HeLa cells, and at the peak of this process 29% of cells can actually be visualized as mitotic, a value generally considered high. Thus the cell cycle points taken around this time would have many cells in late G2 or early G1. The nocodazole experiment of Fig. 6 shows that MCM proteins 6 and 7 are together throughout mitosis, but such experiments do not provide information regarding the presence of MCM proteins on the chromatin. Therefore, to determine whether an MCM protein complex is present on chromatin during mitosis, we have examined MCM7 staining directly in cells that can be visually identified as in metaphase. Tissues were obtained from patients with cervical (Fig. 7A) or ovarian (Fig. 7B) cancer, treated with anti-MCM7 antibody and with secondary antibody coupled to horseradish peroxidase for DAB staining (red-brown). Numerous tumor cells can be seen darkly staining for MCM7, which is largely nuclear. Cells indicated by arrows are in metaphase. The blue hematoxylin counterstain can be seen clearly on the condensed chromosomes of the mitotic cells. These chromosomes are nearly devoid of the MCM7 stain. In addition, the mitotic cells possess only slight, cytoplasmic brown staining, suggesting that MCM7 levels are lower overall in these cells.

**MCM Proteins 4, 6, and 7 Each Decrease in Intracellular Level in Mitosis**—The same nocodazole block used for Fig. 6 was also used for studies of intracellular levels of several MCM proteins, Cdc6 and ORC1, presented in Fig. 7C. For these studies the amounts of extract loaded in gel lanes were normalized to the number of cells. It can be seen that up until the nocodazole block MCM proteins 4, 6, and 7 are each present at significant levels. Upon release from blockage, however, each of these proteins is diminished in level. MCM4 is seen as several bands ranging upward from about 97 kDa, in keeping with observations that it is post-synthetically modified (21). It is notable that overall levels of MCM4 are high throughout S and G2 (Fig. 7), times when MCM4 does not effectively coimmunoprecipitate with MCM6 (Fig. 6). The graph in Fig. 6 reveals that cell division occurs 2–4 h after release from nocodazole. This is in part due to the block by nocodazole early in mitosis, prior to chromosome condensation. After release from nocodazole, the cells must re-establish microtubule structure before proceeding with mitosis. MCM7 levels decline significantly 2–4 h after release, reaching a minimum as cell number becomes nearly double. This is consistent with the lower levels of MCM7 visualized in metaphase cells in Fig. 7 (A and B), because the time between metaphase and cell division is generally quite short. Taken together, the data of Fig. 7 (A–C) indicate that MCM7 is released from chromatin in the later stages of mitosis. At the times when levels of MCM proteins 4, 6, and 7 are lowest (Fig. 7C), the MCM4,6,7 complex is beginning to reconstitute (Fig. 6). Taken together with the chromatin localizations of Figs. 1B and 3A, the data of Fig. 7 indicate that there is a short window of time at mitosis during which MCM7 is absent from chromatin. Before and after this time, as at 10 and 12 h in Fig. 1B, MCM7 is on chromatin and broadly distributed.

It is interesting to compare intracellular levels of MCM proteins 4, 6, and 7 with those of MCM proteins 2 and 3 and with those of Cdc6 and ORC1. MCM3 levels are slightly diminished upon release from nocodazole and remain lower through cell division. In contrast, MCM2 levels are diminished in the presence of nocodazole, but they recover during cell division. Cdc6 levels are high during S-phase and decrease prior to nocodazole.
begin to co-ChIP on the segment at 17.5 h after release from the double thymidine block, and they peak together at 20 h. Interestingly, the two proteins are not detected together at t = 0, which represents cells blocked at the beginning of S-phase. Thus, the co-ChIP of ORC1 and MCM7 at 20 h most likely represents late S-phase cells rather than G2-phase cells, even though the synchrony of the HeLa cells is beginning to break down at this point. A comparison of Fig. 8 with Fig. 7 is informative. ORC1 is present in early-S-phase cells (2 h, Fig. 7), but it is not specifically associated with MCM7 on DNA at this time (Fig. 8). ORC1 begins to appear in the cell cycle upon cell division (Fig. 7), but it is only specifically associated with MCM7 on DNA several hours later (Fig. 8). Cdc6 and MCM7 co-ChIP together earlier in G1 (Fig. 3) than do ORC1 and MCM7 (Fig. 8), but Cdc6 remains specifically associated with MCM7 in early S-phase (Fig. 3). These results indicate that Cdc6 does not completely displace ORC1 from the DNA prior to the loading of MCM7, but they also indicate that Cdc6 and MCM7 remain specifically associated with DNA after ORC1 has departed the complex.

**DISCUSSION**

Alterations in MCM Protein Associations in the HeLa Cell Cycle—MCM proteins 4, 6, and 7 are part of a complex consisting of MCM proteins 2–7, which is essential for initiation of DNA replication in a variety of eukaryotes (2–4, 6). The subcomplex of MCM proteins 4, 6, and 7 reportedly possesses DNA helicase activity (18–20, 44, 45). Although this complex may represent the primary replicative helicase during S-phase, further evidence must be obtained to indicate that it acts exclusively so or that additional proteins are not required for such activity. In any case, MCMs 4, 6, and 7 act on DNA as a hexameric ring structure consisting of two heterotrimers (46), a structure characteristic of DNA helicas throughout evolution. In Fig. 5 we show that MCM7 is coimmunoprecipitated very effectively with anti-MCM4, and in Fig. 5C we show that double ChIP with antibodies to MCM7 and MCM4 reveals specific binding of these two proteins to the 198-bp DNA segment similar to that observed with double ChIP using antibodies to MCM7 and Cdc6. Fig. 6 reveals that MCM6 and MCM7 coimmunoprecipitate with each other at a near-constant level throughout the cell cycle. In contrast, MCM4 does not coimmunoprecipitate with these proteins in late S- and G2-phases (Fig. 6), times when MCM4 intracellular levels remain high (Fig. 7). At these times MCM4 may participate in processes distinct from those of MCM6 and MCM7. Presently, however, we cannot rule out the possibility that extensive modifications of MCM4 may render it less accessible to antibody detection in late S- and G2-phases. We did not detect significant MCM7 on chromatin in metaphase (Fig. 7, A and B). Intracellular levels of MCMs 4, 6, and 7 remain significant after release from nocodazole through the early stages of mitosis, i.e. at times T1' and T2' in Fig. 7. Levels of each decrease upon cell division. Taken together, the immunohistochemical and immunoblotting data indicate that there is a time late in mitosis when MCM 4, 6, and 7 levels are greatly diminished and when MCM7 is not present on chromosomes. Levels of MCM proteins 2 and 3 are altered differently from MCM proteins 4, 6, and 7 during mitosis. MCM3 levels are reduced slightly as the cell number doubles. MCM2 levels are high in S-phase, remain so in G2, and are greatly reduced in the presence of nocodazole. MCM2 levels, in contrast to those of MCM proteins 4, 6, and 7, recover at times T = 3' and T = 4'.

MCM7 Localizes Specifically within an Initiation Zone Upstream of the c-MYC Gene in G1-Phase—It can be seen in Fig. 1 that at 2 and 16 h after release from the double thymidine block, times that represent early S-phase and G1-phase, respec-

**Fig. 6.** Coimmunoprecipitation of MCM proteins 4, 6, and 7 in cells subject to nocodazole block. HeLa cells were synchronized by release from double thymidine block (DTNB) at T = 0 as for previous figures and as described under “Experimental Procedures.” At 8 h after release (T8), the microtubule-disrupting agent nocodazole was added as described under “Experimental Procedures” to block chromosome condensation and thus ensuing mitosis. Four h later, at T12, cells were washed to remove nocodazole and placed in new medium. Times denoted with a prime symbol indicate hours after release from nocodazole block. Filled circles in the graph at the bottom indicate cell numbers corresponding to the gel lanes located directly above them. For the immunoblots shown, 3 µg of anti-MCM6 antibody was used for all immunoprecipitations from cell extracts, and the amounts of protein loaded onto gels were not normalized for cell number. AS denotes asynchronous HeLa cells just prior to the thymidine block. T = 0 DTB Rel denotes a point taken immediately after release from the double thymidine block. T8 Add Noc denotes the point at which nocodazole was added. T12 Rel Noc T = 0' denotes the point at which nocodazole was removed.

Addition. They appear to recover slightly after release from nocodazole, and they are diminished again upon cell division. ORC1 varies dramatically during the cell cycle. Its levels are high in early S-phase but decrease to be virtually undetectable in late S and G2. They remain low in the presence of nocodazole, but they recover after release during cell division.

ORC1 Specifically Colocalizes with MCM7 to the 198-bp Segment in G1-Phase—To help assess the functional significance of specific loading of MCM proteins onto DNA at the G1/S boundary, a double ChIP experiment was performed using anti-MCM7 and anti-ORC1 antibodies. In various non-mammalian systems thus far analyzed, it has generally been found that the presence of an ORC complex on DNA precedes the loading of MCM proteins (3). It has recently been reported that, whereas other ORC proteins remain at constant levels during the cell cycle, ORC1 levels vary (25). Fig. 8 presents results of a cell cycle analysis in which ChIP was performed first with anti-MCM7 antibody and then with anti-ORC1 antibody. The two primer sets of Fig. 1 were used for PCR in this study. Fig. 8 shows great specificity of ORC1 colocalization with MCM7 in two categories: the two proteins are present together only in late G1-phase, and they are present together only on the 198-bp segment and not on the 350-bp segment. The two proteins are present together only on the 198-bp DNA segment located directly above the gel lane located directly above them.
tively, MCM7 is localized primarily to the 198-bp band. This high degree of specific segment isolation via ChIP probably reflects to a certain extent an efficiency of immunoprecipitation based on MCM protein to DNA ratio, the ratio being highest for the smaller, specific segment containing the site of localization. This would in part explain why the 876-bp band is less well represented at 2 and 16 h even though it includes the 198-bp band. The cell cycle experiment of Fig. 1B is internally controlled due to the simultaneous amplification of all three segments. At later time points in late S, G2, and early G1 the 350- and 876-bp bands are very well represented relative to the 198-bp band. The following conclusion can thus be drawn: in G1 and early S-phases MCM7 is specifically localized to a segment centered at 1.7 kb upstream of the c-MYC gene. In late S and G2 it distributes outward from this segment to localize broadly in chromatin. An important feature of Fig. 1B is that, as MCM7 disseminates from its initial localization site, it does not actually leave this site. As can be seen in Fig. 1B, MCM7 is present throughout the cell cycle on its initial 198-bp localization site.

**Fig. 7.** Details of levels of MCM proteins, Cdc6, and ORC1 in cells subject to nocodazole block. MCM protein presence was examined on mitotic chromosomes by immunohistochemistry and in total cell lysates by immunoblotting. MCM7 is not present on chromatin during mitosis in ovarian and cervical cancer cells (A and B). A, staining of a cervical cancer specimen with mouse monoclonal anti-MCM7, seen as red-brown, employed a diaminobenzidine system as described under “Experimental Procedures.” Counterstaining (blue) was with hematoxylin. The micrograph was originally taken at ×400. B, specimen from an ovarian tumor. The micrograph was originally taken at ×200. Lines represent 10 μm. C, levels of MCM proteins 2, 3, 4, 6, and 7 as well as Cdc6 and ORC1 in the HeLa cell cycle at time points before and throughout mitosis. HeLa cells in spinner culture were synchronized using a double thymidine block and blocked after 8 h at metaphase with nocodazole as described under “Experimental Procedures” and the legend to Fig. 6. Cells were from the same experiment as that in Fig. 6, and the graph at the bottom of Fig. 6 is equally applicable to this figure. Total cell lysate was loaded onto an SDS-polyacrylamide gel after normalizing for cell number, 0.4 × 10^6 cells per lane. Immunoblot analysis was then performed using primary antibodies to the indicated proteins as shown.

**Fig. 8.** ChIP with anti-MCM7 and re-ChIP with anti-ORC1 of sequences upstream of the c-MYC gene in the HeLa cell cycle. Double thymidine block was used to synchronize cells as for Fig. 3. ChIP was performed using anti-MCM7 antibody as described under “Experimental Procedures.” Prior to reversal of formaldehyde cross-linking, precipitates were washed, resuspended, and subjected to re-ChIP using anti-ORC1 antibody. PCR was then performed as described for Fig. 3 to analyze presence of 198- and 350-bp segments upstream of the c-MYC gene as described in Fig. 1C.
as MCM7 distributes outward, new MCM7 molecules replace the ones previously located at the initial site. The second possibility is that MCM7 molecules do not migrate from their initial site and that new MCM7 molecules are recruited to effect distribution to multiple sites in chromatin. Our experiments help distinguish between these possibilities.

Timing of Dissemination of MCM7 Throughout the c-MYC Locus Is Consistent with a Slower Recruitment to Replicated DNA Rather than with Replication Fork Progression—In assessing the potential distribution of MCM proteins with replication forks, the overall rate of fork progression must be considered. In Fig. 1B it can be seen that MCM7 is present on the 198-bp segment within 2 h of release from the double thymidine block. In Fig. 2 it can be seen that MCM7 peaks on the 199-bp segment (segment a) about 500 bp upstream of the 198-bp segment, at 8–10 h, whereas MCM7 peaks on the 219- and 203-bp segments (segments b and c) about 4 kb upstream or downstream, at 4–8 h. MCM7 is similarly disseminated to segment d. MCM7 is present on segment e throughout the cell cycle, although at 60 kb away, this segment may be closer to another replicon. Any potential fork pausing, which could explain this asymmetric timing of localization, is considered unlikely due to the great length of time needed to progress through these relatively short DNA lengths. By 8 h nearly all the HeLa cell DNA is replicated (Fig. 1A). Because eukaryotic DNA polymerases generally progress at >100 bp/s (47), these observed peaking times would not be consistent with simple progression with a replication fork. In addition, although MCM7 is distributed widely onto chromatin during S-phase, the rates of ChIP progression observed here are not consistent with progression during active DNA replication. Instead, the rates observed indicate an orderly but much slower recruitment to multiple sites on replicated DNA. These data do not rule out distribution of MCM7 with replication forks, which may occupy a percentage of MCM7 molecules. The data, however, suggest that the bulk of MCM7 molecules are recruited to multiple sites to participate in processes subsequent to DNA replication. These processes may involve functions of MCM proteins similar to those known to be involved in DNA synthesis, functions that could include unwinding at stalled forks or aspects of recombination/repair. MCM proteins may also participate in gene transcription (27, 28). There is evidence that MCM7 plays a role in autoregulation of its own gene expression (28). A new member of the MCM protein family has now been discovered, MCM8, which exists exclusively in metazoan organisms (37, 48). MCM8 evidently plays a protective role against development of neoplasia (37). It is interesting to speculate that demonstrated interaction of MCM8 with MCM4,6,7 (37) alters the function of the latter complex and may play a role in any function of the MCM complex late in the cell cycle.

A Fundamental Difference between ChIP of MCM7 and Double ChIP of MCM7 with Cdc6 during S-phase Sheds Light on Recruitment of MCM7 to Chromatin—A comparison of Fig. 1B with Fig. 3A is highly illuminating. In Fig. 1B MCM7 is specifically localized to the 198-bp band in G1 and early S. In Fig. 3A MCM7 and Cdc6 together are also highly localized to the 198-bp band in G1 (17.5 h) and early S (2.5 h). The difference is that MCM7 alone (Fig. 1B) remains on the 198-bp segment during late S and G2, whereas Cdc6 together with MCM7 (Fig. 3A) is minimally present on the 198-bp segment as the complex is distributed to the 350-bp segment in late S and G2. A logical explanation of the data is that MCM7 is recruited during S-phase to sites distal from the initial late-G1/early-S localization site. As MCM7 remains on the 198-bp segment during S and G2 (Fig. 1B), Cdc6 dissociates from MCM7 at this location (Fig. 3A) and is together with MCM7 primarily at the distal 350-bp site (Fig. 3A). The role of Cdc6 is to load the MCM7, and presumably other MCM proteins, onto the chromatin at the distal sites. The data taken together suggest that new MCM molecules are recruited to sites distal from the initial, pre-replication loading site, i.e. the 198-bp segment, during S-phase and that Cdc6 is involved in loading the new MCM proteins onto the chromatin.

MCM7 Is Released from Chromosomes at a Late Stage in Mitosis—Fig. 1 does not reveal a large change in level of MCM7 on DNA from 10 to 12 h after release from the double thymidine block, a period during which mitosis occurs. Fig. 7 (A and B), however, visually show mitotic chromosomes with little or no MCM7 staining in cells with what appear to be lower levels of MCM7. Fig. 7C indicates that intracellular MCM7 levels remain high during nocodazole block and the early stages of mitosis and decline as cells divide. Taken together, the data indicate that MCM7 is removed from chromatin, and declines in level in cells, during a late stage of mitosis. Either late in mitosis or just after cell division an MCM4,6,7 complex can be detected by coimmunoprecipitation (Fig. 6). There is evidently a short window of time when MCM7 is diminished in chromatin, because it appears on DNA again in early G1 (Fig. 1). It has been reported that in human cells an MCM protein complex forms on DNA late in mitosis (17). That could be consistent with the present findings if the short window of time during which MCM7 is absent does not extend to the end of mitosis. The appearance of MCM proteins on chromatin appears to precede the time at which MCM7 is specifically loaded onto DNA with Cdc6, which occurs later in G1 and early S (Fig. 3). It would be of interest, therefore, to determine whether a complex, containing MCM proteins 4, 6, and 7, forms, with possibly other MCM proteins, prior to their loading onto DNA. Comparison of Figs. 1 and 3 suggests that MCM7 is present on DNA in a more diffuse fashion after mitosis and prior to its site-specific loading with Cdc6. Further work must be done to fully elucidate the potentially complicated loading process.

Specific Association of ORC1 and MCM7 with DNA in Late G1-phase—We report here dynamic changes in ORC1 levels in the HeLa cell cycle. Furthermore, we report that ORC1 and MCM7 simultaneously associate with a specific DNA segment in late G1-phase of the cell cycle. Our results may be compared with recent results of others working with mammalian systems. One group has reported that anti-MCM protein antibodies immunoprecipitate HeLa cell G1-phase chromatin enriched for DNA containing a replication origin. As we do, this group further reports that MCM proteins are distributed to multiple sites during S-phase (24). Our results are similar to those of a group that employed T16 to impose mitotic block in HeLa cells (25). In both cases ORC1 was at minimal levels in cells during such blockage. That group found that ORC1 was associated with ORC proteins 1–4 at the G1/S boundary, a time at which we find ORC1 associated with MCM7. Both studies are consistent with a key role for ORC1 in regulating the status of the pre-replication complex in the cell cycle.

Characteristics of the c-MYC Region to Which MCM Proteins Specifically Localize Prior to Replication—The 198-bp segment to which MCM7 specifically colocalizes, via ChIP, with ORC1, MCM4, and Cdc6 has certain distinguishing characteristics. It is near the center of an initiation zone of DNA replication originally mapped by PCR-based methods (29). Although the c-MYC locus may contain additional centers of bidirectional DNA replication, this particular zone center has been well characterized among mammalian systems. Segments centered either on or near this region reportedly support initiation in chromosomal DNA (29, 31), in plasmids in transfected cells (30), and in a model in vitro system (32). The 198-bp segment con-
tains multiple A-T-rich elements similar to yeast ARS consensus sequences. It also contains a region of potential z-DNA and a prominent site of intrinsic DNA bending (40). In addition it contains a PUR element, i.e. a recognition element for binding the single-stranded DNA-binding and DNA-unwinding protein Puro (40). It is not known whether any DNA helical distortions would facilitate the loading of MCM proteins on to chromatin. Intriguingly, MCM7 has been found, using a yeast two-hybrid system, to associate with the retinoblastoma protein, Rb, an interaction that inhibits DNA replication (49). The hypophosphorylated form of Rb, the form predominant in early G1, also associates strongly with Puro (50). Genetic inactivation in the mouse reveals that Pur

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