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Human Mcm proteins at a replication origin during the G_1 to S phase transition

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ABSTRACT

Previous work with yeast cells and with *Xenopus* egg extracts had shown that eukaryotic pre-replication complexes assemble on chromatin in a stepwise manner whereby specific loading factors promote the recruitment of essential Mcm proteins at pre-bound origin recognition complexes (ORC with proteins Orc1p–Orc6p). While the order of assembly—Mcm binding follows ORC binding—seems to be conserved in cycling mammalian cells in culture, it has not been determined whether mammalian Mcm proteins associate with ORC-bearing chromatin sites. We have used a chromatin immunoprecipitation approach to investigate the site of Mcm binding in a genomic region that has previously been shown to contain an ORC-binding site and an origin of replication. Using chromatin from HeLa cells in G_1 phase, antibodies against Orc2p as well as antibodies against Mcm proteins specifically immunoprecipitate chromatin enriched for a DNA region that includes a replication origin. However, with chromatin from cells in S phase, only Orc2p-specific antibodies immunoprecipitate the origin-containing DNA region while Mcm-specific antibodies immunoprecipitate chromatin with DNA from all parts of the genomic region investigated. Thus, human Mcm proteins first assemble at or adjacent to bound ORC and move to other sites during genome replication.

INTRODUCTION

Mcm proteins were originally discovered in yeast as functions required for the autonomous replication of extrachromosomal DNA elements (Mcm, minichromosomal maintenance). They were subsequently found in all eukaryotes examined and in archaea (reviewed in 1–4). The proteins are required for the initiation of DNA replication and may also be involved in replicative chain elongation (5–10). In addition, some Mcm proteins interact with transcription factors and may therefore function in transcriptional regulation (11–14).

Mcm proteins (Mcm2p–Mcm7p) are divergent in most of their amino acid sequences, but share an approximately 200 amino acid long central region with similarities to a nucleotide-binding fold that includes variations of the Walker A and Walker B motifs, as are found in other members of the large AAA^+ family of proteins (ATPase associated with various cellular activities) (15,16). In addition, Mcm2p, Mcm4p, Mcm6p and Mcm7p possess a zinc finger region of the type $CX_2CX_nCX_2C$ that may be involved in protein–protein interactions (17). In extracts from yeasts, mammalian cells and *Xenopus* eggs, Mcm proteins occur in defined subcomplexes such as stable Mcm3p–Mcm5p dimers and single or double Mcm4p–Mmc6p–Mcm7p trimers as well as single or double hexamers containing all six Mcm proteins (18–24). However, the functional complex *in vivo* is not yet known.

Mcm proteins are loaded on chromatin at the end of mitosis and the beginning of the G_1 phase of the cell cycle. Work with yeast cells has shown that Mcm loading is contingent upon the presence on chromatin of the six subunit origin recognition complex (ORC with subunits Orc1p–Orc6p) and depends on Cdc6p which interacts with ORC (25–29). Biochemical experiments with *Xenopus* egg extracts support this scheme showing that ORC must first be present on chromatin, followed by the binding of the *Xenopus* homolog of Cdc6p and of another Mcm-loading factor, Cdt1p (also known as RLF-B), before Mcm proteins are recruited to complete the formation of pre-replication complexes on chromatin (9,22,30–33). It is quite likely that all eukaryotes use the same general pathway for the assembly of pre-replication complexes and the formation of replication-competent chromatin (34,35).

The conversion of pre-replication complexes into active replication complexes at the G_1/S phase transition depends on the activities of cyclin-dependent kinases (CDK2 with cyclin A or cyclin E in mammalian cells) and of the Dbf4/Cdc7 kinase (reviewed in 2).

During S phase, Mcm proteins are gradually released from their chromatin sites (26,36–39). Their reloading appears to be prevented by several mechanisms, including the function of the S phase-specific protein geminin that binds to and neutralizes the function of the loading factor Cdt1p (40–43). This constitutes a powerful mechanism preventing the re-replication of chromatin sections that have already replicated during the same S phase.

The molecular functions of Mcm proteins on replicating chromatin *in vivo* are not fully understood. The conserved nucleotide-binding fold suggests that ATP binding and ATP hydrolysis are important for the replication functions of Mcm proteins (44,45). Indeed, ATP stabilizes the interaction of

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Mcm proteins with isolated chromatin (21,46). Importantly, the mammalian Mcm4p–Mcm6p–Mcm7p trimer has been reported to possess ATPase and DNA helicase activity *in vitro* (47–49), as does a hexameric archaeal protein related to the Mcm2p–Mcm7p family (50–52). Furthermore, *in vivo* crosslinking and chromatin immunoprecipitation (ChIP) experiments have shown that yeast Mcm proteins are associated with origin sequences in pre-replication complexes, but appear to move with replication forks after initiation, as expected for a DNA helicase (5). A participation of yeast Mcm proteins in replicative chain elongation is strongly supported by an elegant study with Mcm 'degron' mutants which allow the precise destruction of individual Mcm proteins during ongoing S phase resulting in a stop of replication chain elongation (8).

ChIP was used to localize Mcm proteins in mammalian systems. It was shown that Mcm proteins are localized to the Chinese hamster DHFR origin region in G_1 cells and partition to more distal parts of the DHFR locus in S phase cells (53). In these experiments, DNA in immunoprecipitated chromatin was radiolabeled and hybridized to dot-blotted large cosmid-borne genome fragments, a procedure that inevitably results in low signal-to-noise ratios and low resolution. Therefore, we found it of interest to investigate Mcm loading by a procedure that involves *in vivo* formaldehyde crosslinking and ChIP to identify the DNA in immunoprecipitated chromatin by quantitative real-time PCR. As shown recently, this procedure allows the localization of ORC-binding sites and replication origins within regions of ~500 bp in human HeLa cell chromatin (54,55). Here we investigate whether Mcm proteins assemble at the ORC-bearing chromatin site. We show that Mcm-specific antibodies preferentially immunoprecipitate specific origin-containing chromatin fragments from G_1 cells, but not from S phase cells where Mcm proteins appear to be distributed over the entire chromatin section investigated. Thus, human Mcm proteins assemble at ORC-binding sites during G_1 phase, but disperse to other chromatin sites during S phase.

MATERIALS AND METHODS

Cell culture and synchronization

Human HeLa S3 cells were grown to semiconfluency on plastic dishes in Dulbecco's modified Eagle's medium plus 5% fetal calf serum.

For S phase synchronization, cells were first subjected to a double thymidine block (56) and then released for 2–4 h. Cells in G_1 phase were prepared in the following manner: they were first arrested by a single thymidine block, released for 9 h and then arrested again by a short treatment (3 h) with nocodazole. Mitotic cells were collected and resuspended in culture medium without nocodazole and investigated at 4 h without nocodazole. Synchronization was verified by analysis of DNA content in a flow cytometer (see Fig. 2).

In vivo crosslinking

Formaldehyde was diluted to 1% in prewarmed medium (37°C) and added to monolayers of ~10^8 cells for 4 min (57). After removal of the medium, cells were washed three times on plates with cold phosphate-buffered saline (PBS) (58), scraped off, washed again twice in cold PBS and then resuspended in hypotonic RSB buffer (10 mM Tris–HCl, 3 mM $MgCl_2$, pH 8.0). All buffers contained 10 mM sodium bisulfite as a protease inhibitor. After 10 min on ice, the swollen cells were disrupted by Dounce homogenization. Nuclear material was collected and washed twice in RSB buffer and once in high salt NSB buffer (1 M NaCl, 10 mM Tris–HCl, 0.1% NP-40, 1 mM EDTA, pH 8.0). Nuclear material was then resuspended in low salt NSB buffer (0.1 M NaCl) and loaded onto a step gradient made up of 1.75, 1.5 and 1.3 g/ml CsCl in 20 mM Tris–HCl, 0.5% sarcosyl, 1 mM EDTA, pH 8. Nucleoprotein complexes were collected after centrifugation (37 000 r.p.m., 24 h, 18°C) and dialyzed overnight against TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.4) containing 10 mM sodium bisulfite. Nucleoprotein was then briefly sonicated on ice and digested with micrococcal nuclease in TE with 3 mM $CaCl_2$ (1 U micrococcal nuclease/100 µg nucleoprotein for 15 min at 37°C) (55).

Chromatin immunoprecipitation

Affinity-purified antibodies against the human Orc2 (59), Mcm (60) and replication protein A (RPA) (large subunit) proteins (61) have been described. The control antibodies were non-specific rabbit IgG from Sigma.

Immunoprecipitations were performed in NET buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40). Nuclease-digested nucleoprotein was centrifuged for 10 min at 15 000 *g* for clarification. Soluble nucleoprotein (1 mg) was incubated with antibodies (10 µg) for 2 h at 20°C. Then, 50 µl of protein A–Sepharose (Amersham Pharmacia Biotech) was added for an additional 2 h. Immunocomplexes were washed eight times with RIPA (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium desoxycholate, 0.1% SDS, pH 8), three times in lithium buffer (10 mM Tris–HCl, 250 mM $LiCl_2$, 0.5% NP-40, 0.5% sodium desoxycholate, 1 mM EDTA, pH 8) and five times in TE buffer. The washed precipitates were divided for western blotting and DNA extraction. For western blot analyses, proteins were eluted from crosslinked chromatin and processed as previously described (60). For DNA extraction, the immunoprecipitates were first washed again as described above to maximize the signal-to-noise ratio. The final pellet was then resuspended in TE with 1% SDS and incubated overnight at 37°C with 200 µg/ml proteinase K. The DNA was purified by standard phenol/chloroform extraction and ethanol precipitation. The precipitated DNA (usually between 5 and 10 ng) was dissolved in 40 µl of TE. One-twentieth was used for real-time quantitative PCR.

Quantitative real-time PCR analyses

Real-time PCR was performed with a Light Cycler instrument (Roche Diagnostics) using a ready-to-use 'hot start' reaction mix (FastStart DNA Master SYBR Green I; Roche Diagnostics). This mix contains *Taq* DNA polymerase and a fluorescent dye, SYBR Green I, for real-time detection of double-stranded DNA. Reactions were set up in 10 µl volumes including 0.5 mM each primer. PCR reactions were performed for 35 cycles routinely using standard settings as recommended by the manufacturer. Annealing temperatures for each primer pair are given in Table 1. Standard DNA samples (human genomic DNA) were serially diluted to 30 and 3 ng and 300, 30 and 3 pg. Following PCR, the *x*-axis crossing

Table 1. Sequences and amplification conditions for primers

Primer	Sequence (5′→3′)	Map positions (bp)	Length (bp)	Annealing temperature (°C)
EX9-F	ATGTCTTCCGGAGACTCCTGAAGC	6342–6365		
EX9-R	GGCCTCCTATTCTCAGAATCATGC	6705–6728	387	62
EX7-F	TAATCCGTCACCTTGACTACCACC	8901–8924		
EX7-R	ACAGCACGTGCATGATTCTGTAGG	9277–9300	400	62
EX6-F	TACCTGTGGGTAAGAGATGAGTTG	10691–10704		
EX6-R	TGCCTGTTCCCAAATGCTATATGC	10998–11021	331	62
EX2-F	TCTGCACTCCGTTCAGCTCCTCTG	11894–11917		
EX2-R	GAGTGAGGATGCCAGGTCATCTCC	12191–12214	321	68
PROM-F	AAACCAGAAGTAGGCCTCGCTCGG	12946–12969		
PROM-R	GGCCAGTAAGCGCGCCTCTTTGG	13460–13482	537	68
IN1-F	ATCTCGCCTAATCCCACCAGTACC	14364–14387		
IN1-R	CATATTCACTACTAGACCCTCCGG	14633–14656	293	62
IN6-F	GACATTCTGCTTCCATAGATGTGG	19943–19966		
IN6-R	GTTGGGAAAGATGTCATCATCAGG	20265–20288	346	62
IN7-F	GAGGAATGCCAGAATTTCCAGAGG	26412–26435		
IN7-R	TTCCATCTGGAATGAGATCCCAGC	26118–26741	327	62

point of each standard sample was plotted against the logarithm of concentration to produce a standard curve. Genomic equivalents of DNA samples were determined by extrapolation from the standard curve (55).

RESULTS

Immunoprecipitations of crosslinked proteins

To determine whether human Mcm proteins are preferentially associated with distinct sites on chromatin *in vivo*, we used a ChIP approach as described before (54,55). We first asked whether the antibodies used in this study recognize Mcm proteins in crosslinked chromatin. For that purpose, chromatin was prepared from proliferating HeLa cells treated for various lengths of time (1–10 min) with formaldehyde (1%). It could be shown that a formaldehyde treatment of 4 min was sufficient to covalently link chromatin-bound Orc2p and Mcm proteins to DNA (data not shown; but see 55). Crosslinked chromatin was treated with micrococcal nuclease to produce nucleoprotein fragments with ~1 kb of DNA. These fragments were then incubated with monospecific antibodies for immunoprecipitation.

As a control, we used Orc2p-specific antibodies that had already been tested in previous experiments and shown to effectively immunoprecipitate chromatin with crosslinked Orc proteins (54,55,60). Interestingly, Orc2p-specific antibodies failed to co-immunoprecipitate Mcm3p (Fig. 1A) even though the chromatin sample investigated contained high amounts of crosslinked Mcm3p (input in Fig. 1B), and a close association of ORC and Mcm proteins could be expected since chromatin binding of ORC is necessary in yeast and *Xenopus* egg extracts for the subsequent loading of Mcm proteins (see Introduction). Results similar to those of Figure 1A were obtained when the blots were probed with antibodies against the other Mcm proteins (data not shown). An interpretation is that Orc2p (and by implication other components of ORC) (62) do not necessarily co-localize with Mcm proteins in proliferating HeLa cells (60).

Using Mcm3-specific antibodies, Mcm3p-bearing chromatin was immunoprecipitated that also contained relatively large amounts of crosslinked Mcm5p, the dimer partner of Mcm3p, but only small amounts of the other Mcm proteins, although they were present in the input sample. Interestingly, the immunoprecipitated chromatin also carried Orc2p, although in relatively small amounts (Fig. 1B).

Likewise, Mcm4-specific antibodies immunoprecipitated chromatin with crosslinked Mcm4p as well as Mcm6p and Mcm7p, components of the stable Mcm4p–Mcm6p–Mcm7p trimer (Fig. 1C, upper panels). A supernatant blot and the precipitate blot were also probed with Mcm3 and Mcm5 antibodies showing that only small amounts of the Mcm3p–Mcm5p dimer co-immunoprecipitated with the Mcm4p–Mcm6p–Mcm7p trimer (not shown). Significantly, however, Mcm4 antibodies (just like Mcm3 antibodies; see above) co-immunoprecipitated some of the crosslinked Orc2p (Fig. 1C, lower panels).

We conclude from Figure 1, first, that Mcm proteins, known to occur as stable subcomplexes in protein extracts (the Mcm3p–Mcm5p dimer and the Mcm4p–Mcm6p–Mcm7p trimer), preferentially crosslink together in chromatin and, second, that Mcm3- as well as Mcm4-specific antibodies co-immunoprecipitate small amounts of Orc2p, whereas Orc2-specific antibodies fail to co-immunoprecipitate Mcm proteins. This could mean that Orc2p and Mcm proteins do not always crosslink to the same chromatin fragment, but, when they do, Orc2p becomes inaccessible to Orc2 antibodies and may be covered by Mcm proteins (which are about 10 times more abundant in nuclei than Orc proteins; 1).

Since the experiments in Figure 1 were performed with asynchronously proliferating HeLa cells, we repeated the ChIP assay with Mcm3-specific antibodies comparing chromatin from cells in G_1 phase with chromatin from cells in S phase (see Material and Methods). The data obtained with G_1 chromatin were similar to those of Figure 1 and showed that immunoprecipitated chromatin carried Mcm3p and Mcm5p in addition to small amounts of Orc2p. However, S phase chromatin, immunoprecipitated with Mcm3 antibodies, contained the Mcm3p–Mcm5p dimer, but lacked detectable

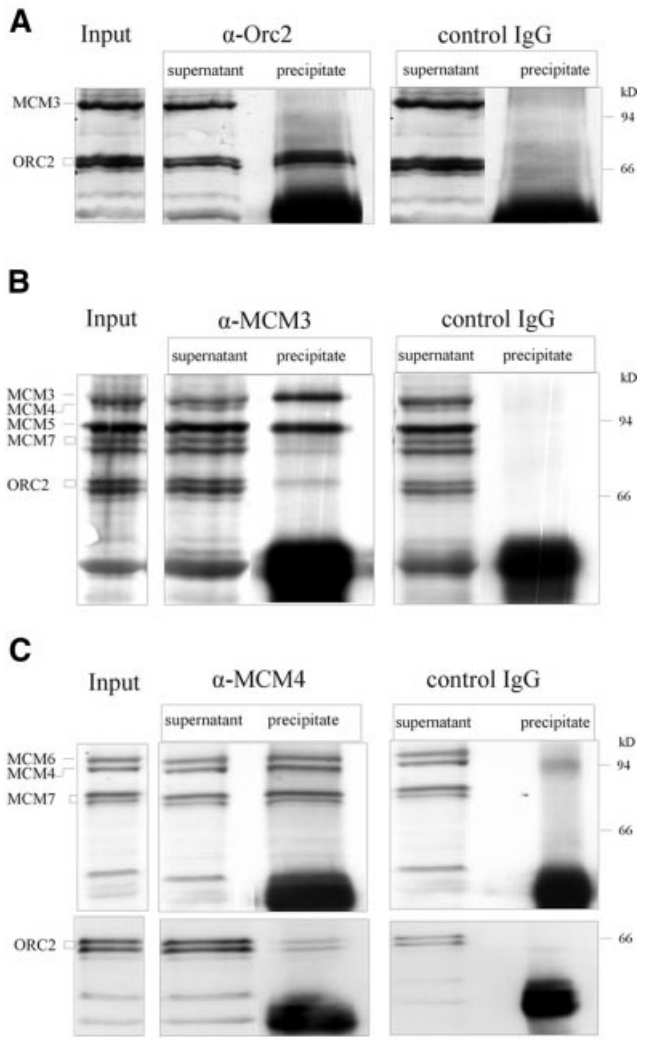


Figure 1. Orc2p and Mcm proteins on crosslinked chromatin. (**A**) Immunoprecipitations with antibodies against Orc2p (α-Orc2) and with non-specific control antibodies (IgG). Input (crosslinked, nuclease-treated chromatin before immunoprecipitation), supernatants (remaining chromatin after treatment with antibodies and protein A–Sepharose) and immunoprecipitates were analyzed in western blots with Mcm3p-specific (MCM3) and with Orc2p-specific antibodies (ORC2). (**B**) Immunoprecipitations with antibodies against Mcm3p (α-MCM3) and with non-specific control antibodies (IgG). The input, supernatant and precipitated samples were western blotted and analyzed with antibodies against Mcm3p (MCM3), Mcm4p (MCM4), Mcm5p (MCM5) and Mcm7p (MCM7) as well as with antibodies against Orc2p (ORC2). (**C**) Immunoprecipitation with antibodies against Mcm4p (α-MCM4) and with non-specific control antibodies (IgG). The input, supernatant and precipitated samples were western blotted and analyzed with Mcm4p, Mcm6p and Mcm7p antibodies as well as with antibodies against Orc2p as indicated.

Orc2p (Fig. 2). Thus, according to ChIP, Mcm proteins and Orc2p may co-localize on G_1 phase chromatin, but not on S phase chromatin.

We include in Figure 2 western blots performed with antibodies against the large subunit of RPA, the major eukaryotic single strand-binding protein with function at replication forks (63). We show that the RPA subunit did not detectably crosslink to Mcm3p–Mcm5p-bearing chromatin fragments indicating that Mcm proteins and RPA are not located at closely adjacent sites on chromatin.

We note though that only a fraction of the total crosslinked chromatin could be immunoprecipitated by Mcm antibodies (Figs 1 and 2 and related experiments). This fraction remained unchanged upon the addition of several-fold higher amounts of Mcm-specific antibodies indicating that a considerable fraction of crosslinked Mcm proteins did not interact with, or were inaccessible to, antibodies. However, Ritzi *et al.* (60) have previously used a different method, which allowed an investigation of total chromatin, namely partial digestion of native chromatin with micrococcal nuclease. They showed that Orc proteins and Mcm proteins usually occur on chromatin digestion products of different size classes and concluded that ORC and Mcm proteins are not frequently bound to neighboring sites in HeLa cell chromatin.

To summarize this section we conclude that a fraction of Mcm proteins on crosslinked chromatin is accessible to specific antibodies and can be immunoprecipitated. We therefore extracted DNA from immunoprecipitated chromatin and used the method of quantitative real-time PCR for the detection of specific DNA sequences.

The *MCM4* gene locus in crosslinked chromatin

The chromatin region investigated was the human *MCM4*/*PRKDC* locus, a region of ~25 kb that includes an ORC-binding site and an origin of replication in an ~500 bp segment between the two divergently transcribed constitutively expressed genes (64) (Fig. 3A).

As has been discussed before (54), DNA from different regions of crosslinked chromatin is not necessarily amplified with similar efficiencies by PCR. One reason for this is that crosslinked proteins such as transcription factors are unevenly distributed in chromatin with the consequence that some regions are better protected than others against micrococcal nuclease digestion, a routine step in the preparation of crosslinked chromatin for immunoprecipitation (see above). We indeed found that promoter-proximal DNA from crosslinked chromatin could be amplified to 2- to 3-fold higher values than promoter-distal DNA in the same preparation (Fig. 3B, input). The different abundance of amplifiable promoter-proximal and promoter-distal parts of the gene region in crosslinked chromatin prior to immunoprecipitation has to be considered when evaluating the PCR results of ChIP assays.

In Figure 3C, we prepared crosslinked chromatin from asynchronously proliferating HeLa cells. Irrelevant control antibodies (control IgG in Fig. 3C) failed to precipitate specific DNA sequences, whereas Orc2-specific antibodies preferentially immunoprecipitated DNA sequences from the intergenic region that includes upstream promoter sequences of the *MCM4* gene (55) (α-Orc2 in Fig. 3C). Interestingly, Mcm3-specific as well as Mcm4-specific antibodies immunoprecipitated chromatin with the upstream promoter site, but also chromatin with DNA regions corresponding to more distal parts of the two divergent genes (Fig. 3C, α-MCM3 and α-MCM4). The distribution of Mcm4-bearing chromatin fragments seems to show a gradient-like pattern with decreased presence of fragments at increased distance from the origin. As detailed below, this distribution is most

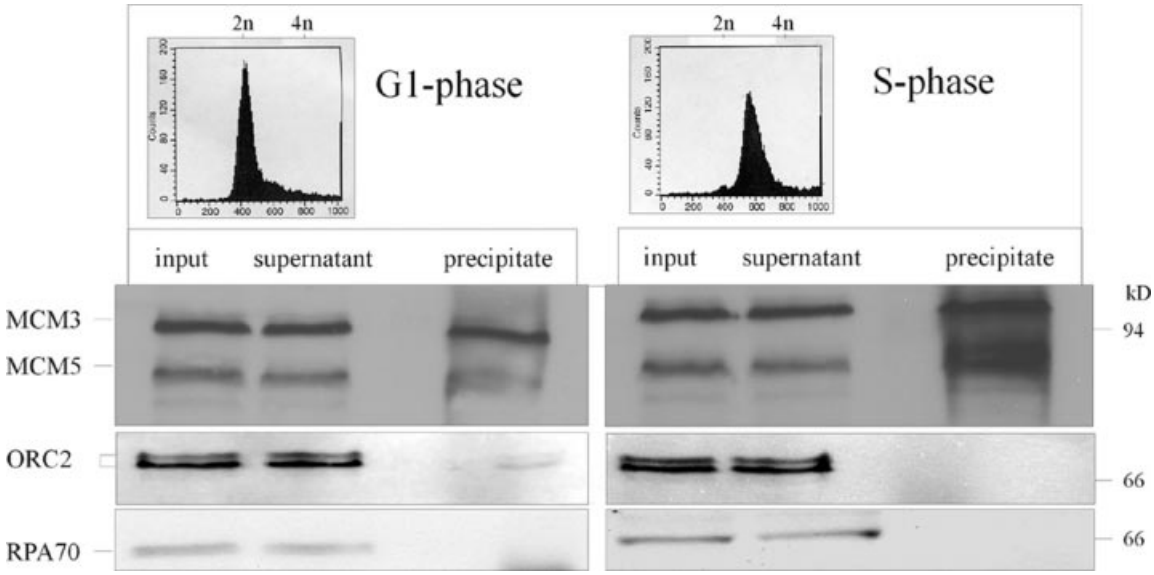


Figure 2. ChIP assay on crosslinked chromatin from synchronized HeLa cells. Crosslinked chromatin from G_1 phase cells and S phase cells was immunoprecipitated with antibodies against Mcm3p (α-MCM3). The input, supernatant and precipitated samples were western blotted and analyzed with Mcm3p, Mcm5p and Orc2p antibodies as well as with antibodies against the large subunit of RPA (RPA70) as indicated. Synchronization was verified by analysis of DNA content on a flow cytometer (shown in the conventional manner).

probably not significant given the fact that the origin-proximal sequences can be PCR amplified to a higher degree than origin-distal sequences.

We noted in this and related experiments (see below) that the number of total PCR-amplifiable sequences were more abundant in Orc2 precipitates than in Mcm3 or Mcm4 precipitates. One explanation is that Orc2-specific antibodies were more efficient than Mcm-specific antibodies in immunoprecipitating crosslinked chromatin, but it is also possible that DNA fragments extracted from Orc2 precipitates are more suitable as templates for PCR than DNA fragments extracted from Mcm precipitates. This could be due, for example, to an incomplete reversal of covalent linkages between protein and DNA or, and probably more likely, to breaks or other discontinuities in the DNA strands of Mcm-bearing crosslinked chromatin.

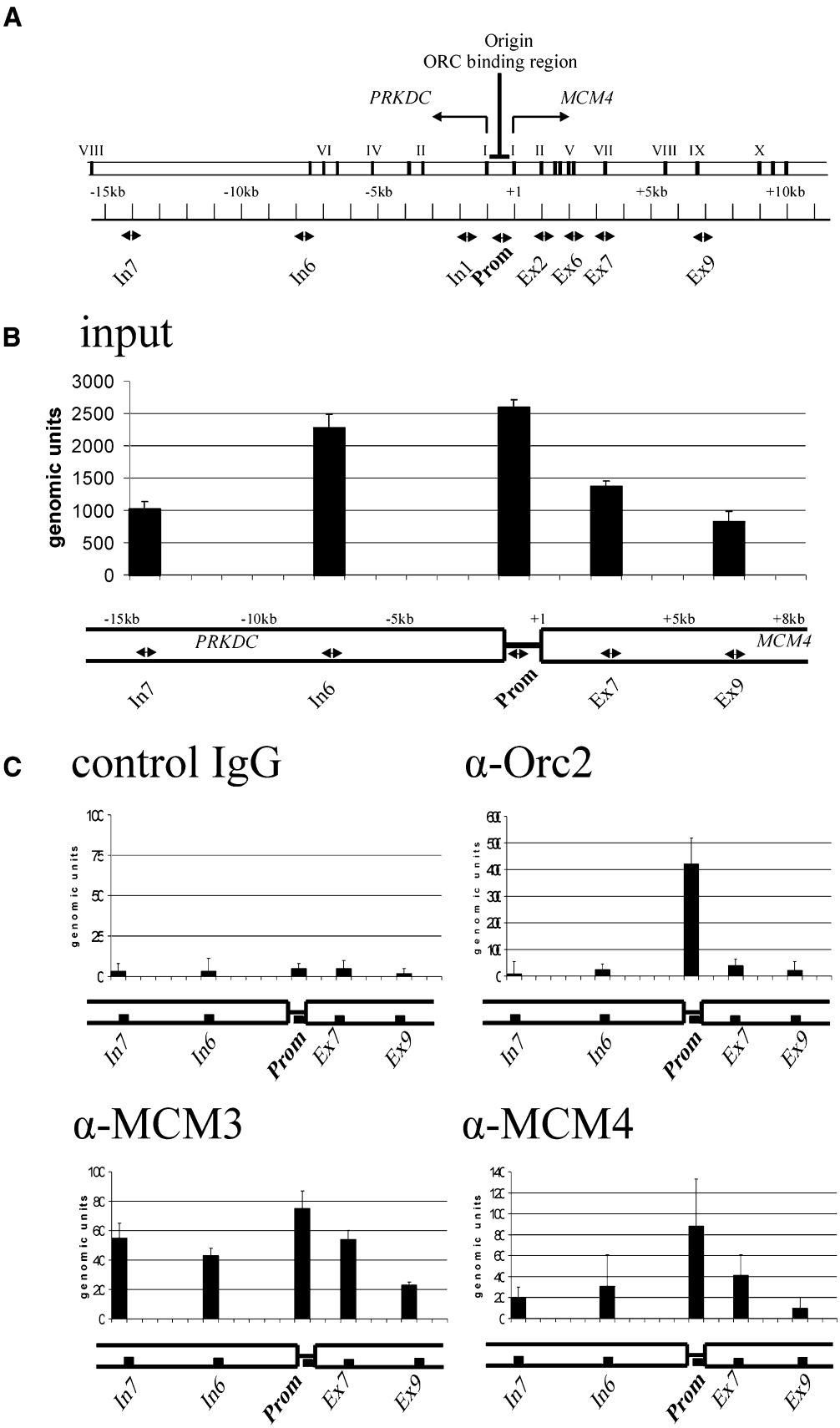
In either case, the data of Figure 3 show that Mcm3- and Mcm4-specific antibodies are able to precipitate chromatin with PCR-amplifiable DNA. We next determined whether the location of Mcm proteins varies during the cell cycle and performed experiments with cells in G_1 phase and compared them with cells that were released into S phase.

Mcm proteins at the origin

We have performed several independent cell synchronization experiments and prepared crosslinked chromatin, both from cells before and after release into S phase (see Materials and Methods and Fig. 2). ChIP assays were performed with samples from each preparation and the abundance of specific DNA regions in the immunoprecpitates was evaluated by quantitative PCR using five primer pairs in some and eight primer pairs in other experiments.

As a summary of the results (Fig. 4) we note the following. First, Orc2-specific antibodies specifically precipitated chromatin fragments with sequences that could be amplified by primers corresponding to the upstream promoter region (Prom) and to the Ex2 region. These two regions are adjacent to each other (distance <1 kb) and could therefore occur on the same chromatin fragments. The results were similar for crosslinked chromatin prepared from G_1 phase cells and from S phase cells (compare left and right panels in Fig. 4A). Second, Mcm3-specific antibodies preferentially immunoprecipitated promoter-proximal sequences of crosslinked chromatin from cells in G_1 phase. In fact, ~75% of the estimated total amplifiable DNA sequences ('genomic equivalents' in Fig. 4) in these precipitates corresponded to promoter-proximal sites (left panel in Fig. 4B). In contrast, immunoprecipitates performed with S phase chromatin yielded only ~30% of amplifiable promoter-proximal DNA. In these immunoprecipitates most amplifiable DNA corresponded to promoter-distal regions (right panel in Fig. 4B). Third, ChIP experiments with Mcm4-specific antibodies gave results that

Figure 3. (Opposite) Specific DNA regions in immunoprecipitated crosslinked chromatin from unsynchronized HeLa cells. (**A**) Genomic organization of the analyzed region encompassing human genes *PRKDC* and *MCM4* (70). In the double line diagram, exons are shown as black boxes and arrows indicate the starts and directions of transcription. In the single line diagram, divergent arrowheads show regions complementary to the PCR primers used. (**B**) Input. DNA was extracted from crosslinked chromatin before immunoprecipitation and amplified by quantitative PCR using the primer sets indicated. The results are expressed in 'genomic units' relative to serially diluted genomic control DNA. (**C**) Immunoprecipitated chromatin. Quantitative real-time PCR with DNA templates extracted from chromatin precipitated with control antibodies and with antibodies against Orc2p, Mcm3p and Mcm4p as indicated.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Origin
ORC binding region
PRKDC
MCM4
VIII
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IV
II
I
I
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VII
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-10kb
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+1
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In7
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3000
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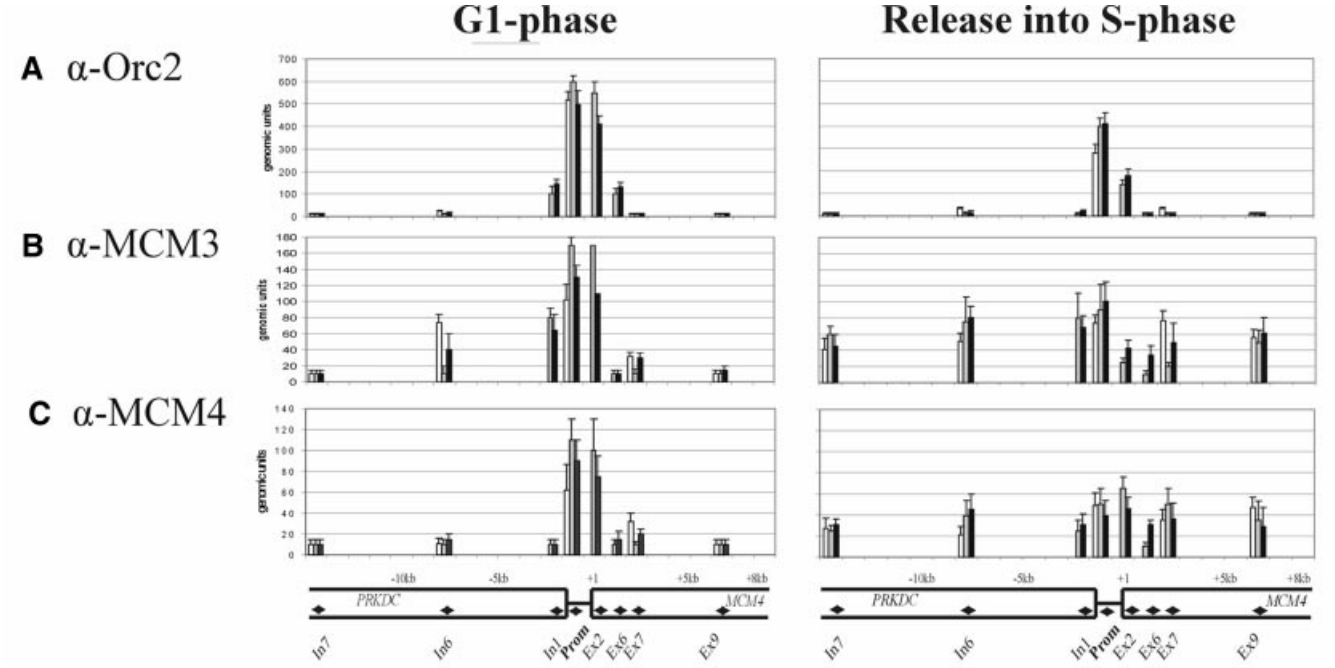

Figure 4. Specific DNA regions in immunoprecipitated crosslinked chromatin from cells in G_1 phase and S phase. HeLa cells were synchronized in G_1 phase (left) or released into S phase for 4 h (right) and were subjected to ChIP analysis. Quantitative real-time PCR was performed with DNA templates extracted from chromatin precipitated with antibodies against (**A**) Orc2p, (**B**) Mcm3p and (**C**) Mcm4p. We show the results of three independent experiments. (Below) Schematic representation of the genomic region analyzed with the PCR-amplified segments indicated by arrowheads.

confirmed those obtained with Mcm3-specific antibodies, showing again that Mcm antibodies preferentially immunoprecipitate chromatin with an ORC-binding site and with a replication origin from G_1 phase cells, and chromatin with promoter-distal sequences from S phase cells (left and right panels in Fig. 4C). We have performed several experiments in addition to those shown in Figure 4. In these experiments, we used Mcm5- and Mcm7-specific antibodies for the immunoprecipitation of crosslinked chromatin. The data obtained were very similar to those of Figure 4 and are therefore not shown. We have also compared chromatin from cells released for 2 h into S phase and found a distribution very similar to that of immunoprecipitated chromatin fragments shown in Figure 4 (data not shown). Thus, a wave of apparently synchronous Mcm protein migration such as that described for yeast elongation (5) does either not occur in HeLa cells (due to an inherent asynchrony in origin activation) or cannot be observed under the present experimental conditions.

As noted above, evaluation of ChIP assays requires a comparison of the PCR results in the immunoprecipitates (Fig. 4) with those in the input crosslinked chromatin (Fig. 3B). Accordingly, we calculated the ratios of amplifiable precipitated over input DNA. The data show, for the Orc2 immunoprecipitates, that promoter-proximal sequences (amplified using primer pairs Prom and Ex2) were 10- to 20-fold enriched over promoter-distal sequences (Fig. 5A). In the Mcm3 and Mcm4 immunoprecipitates of chromatin from G_1 phase cells, promoter-proximal sequences were also enriched, although more moderately (2- to 6-fold), over promoter-distal sequences (open squares in Fig. 5B and C). The preference for promoter-proximal sites was lost in immunoprecpitated chromatin from S phase cells where all regions in the *MCM4*/*PRKDC* gene locus appeared to be present in approximately similar copy numbers (closed circles in Fig. 5B and C).

DISCUSSION

We used the ChIP technology to investigate the location of components of the pre-replication complex in the human genome. We previously identified prominent ORC-binding sites within ~500 bp regions upstream of several constitutively expressed genes. These sites usually coincide with origins of replication as determined by the nascent strand abundance assay (54,55).

We have now used ChIP to determine the position of Mcm proteins on chromatin. This is of interest because work with cycling mammalian cells has shown that the binding of Mcm proteins to chromatin follows the binding of ORC, but, unlike the situation in yeast and *Xenopus* egg extracts, it has not been determined whether human ORC is required for the subsequent binding of Mcm proteins, nor is it known whether Mcm proteins assemble at ORC. While an effective *in vitro* system will be necessary to investigate the first point, our data contribute to the second point as they show that Mcm proteins preferentially occur at the ORC-binding region in G_1 phase cells.

In our experiments, we used monospecific antibodies against Mcm3p, a component of the stable Mcm3p–Mcm5p dimer, and antibodies against Mcm4p, a member of the

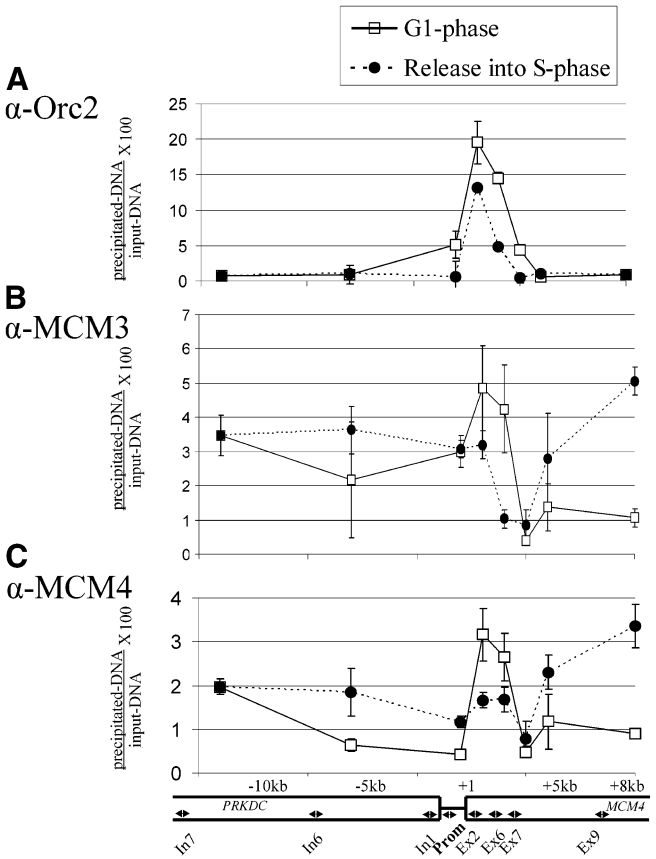

Figure 5. Enrichment of specific DNA sequences in immunoprecipitates. Quantitative PCR gives the results in 'genomic units' relative to serially diluted purified genomic DNA. The ratios of precipitated over input genomic units are multiplied by 100 and plotted against the primer sites on the analyzed region. The results of the precipitations with Orc2 antibodies are summarized in (**A**), with Mcm3 antibodies in (**B**) and with Mcm4 antibodies in (**C**). Open squares indicate the mean enrichment of the G_1 phase samples and closed circles indicate the mean enrichment of the samples from S phase cells.

Mcm4p–Mcm6p–Mcm7p trimer that has been reported to function as a helicase *in vitro* (47,48,65,66). Immunoprecipitation results suggest that the dimer and trimer complexes seem to crosslink independently of each other (Fig. 1). However, they crosslink to the same DNA sequences, at least in the genomic region investigated, and it is therefore likely that they function together *in vivo*. For that purpose, we do not distinguish between Mcm3 and Mcm4 immunoprecipitates in the following discussion.

It became clear from this and earlier studies that Orc2p and possibly other Orc proteins remain bound to specific chromatin sites during the cell cycle (with the exception of Orc1p, which dissociates from chromatin and is degraded during the S phase of cycling mammalian cells; 59,67). We now show that Mcm proteins associate in G_1 phase with the ORC-bearing chromatin sites.

While this conclusion is in agreement with the generally accepted scheme of pre-replication complex formation in eukaryotes, we made some observations that should be commented on.

One observation is that Orc2p-specific antibodies precipitate chromatin that carries Orc2p, bound to its upstream promoter site, but lacks Mcm proteins. Thus, a number of ORCs may not be in contact with Mcm proteins. This was not only found in ongoing S phase when ORC remains bound while Mcm proteins move away from the origin (and eventually dissociate from chromatin), but also in G_1 phase. This could mean that some ORC do not recruit Mcm proteins and may remain silent during that particular cell cycle.

A second point to consider is that Mcm-specific antibodies precipitate chromatin, which, in addition to high amounts of Mcm proteins, also carries Orc2p, although in relatively small amounts. As already mentioned, the fact that Mcm antibodies co-immunoprecipitate Orc2p but Orc2 antibodies fail to co-immunoprecipitate Mcm proteins can be explained by assuming that Mcm proteins cover chromatin-bound Orc2p which thus becomes inaccessible in these complexes. That Orc2p and Mcm proteins indeed occur at identical chromatin sites was shown by PCR analyses of DNA extracted from immunoprecipitated chromatin from G_1 phase cells.

In this regard we note that Mcm antibodies precipitate a considerable fraction of chromatin with crosslinked Mcm proteins (Fig. 1). However, PCR analyses resulted in rather small numbers of Mcm-associated amplifiable sequences, at least relative to Orc2-associated sequences, which are detected in 3- to 4-fold higher copy numbers in immunoprecipitated chromatin (Figs 3 and 4) even though Orc proteins are clearly less abundant in chromatin than Mcm proteins (1). Thus, Mcm-bearing DNA extracted from immunoprecipitated chromatin cannot be PCR amplified to similar extents as ORC-bearing DNA in the same preparation. This could be caused by structural features of the Mcm-bearing DNA such as single-strand regions or strand discontinuities. In fact, it has been described that chromatin with associated Mcm proteins is more readily attacked by micrococcal nuclease than bulk chromatin or chromatin with Orc proteins, suggesting that Mcm proteins normally reside in more open chromatin regions where the underlying DNA may undergo specific transitions such as helix unwinding in S phase (60,68).

This interesting point certainly deserves further investigation. However, with the present experimental approach we detect only those Mcm-bearing DNA segments that can be amplified in PCR analyses. With these reservations in mind, we conclude that Mcm proteins preferentially occur at an origin site before S phase, but distribute over more distal parts of the genes during ongoing S phase. This is similar to previous results with yeast cells (5,69) and could mean that Mcm proteins are migrating in both directions from the origin with the divergently moving replication forks, as expected if Mcm proteins constitute the replicative DNA helicases in eukaryotic cells.

However, according to biochemical experiments, only the Mcm4p–Mcm6p–Mcm7p trimer functions as a DNA helicase *in vitro* whereas the Mcm3p–Mcm5p dimer and the hexameric Mcm complex are enzymatically inactive (47–49). We suggest now that Mcm3p–Mcm5p dimers and Mcm4p–Mcm6p–Mcm7p trimers migrate together with replication forks and may therefore functionally interact *in vivo*, although it is presently unclear what their combined function could be.

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