A Potential Role for Mini-chromosome Maintenance (MCM) Proteins in Initiation at the Dihydrofolate Reductase Replication Origin

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Mini-chromosome maintenance (MCM) proteins were originally identified in yeast, and homologues have been identified in several other eukaryotic organisms, including mammals. These findings suggest that the mechanisms by which eukaryotic cells initiate and regulate DNA replication have been conserved throughout evolution. However, it is clear that many mammalian origins are much more complex than those of yeast. An example is the Chinese hamster dihydrofolate reductase (DHFR) origin, which resides in the spacer between the DHFR and 2BE2121 genes. This origin consists of a broad zone of potential sites scattered throughout the 55-kb spacer, with several subregions (e.g. ori-β, ori-β’), and ori-γ being preferred. We show here that antibodies to human MCM2-7 recognize counterparts in extracts prepared from hamster cells; furthermore, co-immunoprecipitation data demonstrate the presence of an MCM2-3-5 subcomplex as observed in other species. To determine whether MCM proteins play a role in initiation and/or elongation in Chinese hamster cells, we have examined in vivo protein-DNA interactions between the MCMs and chromatin in the DHFR locus using a chromatin immunoprecipitation (ChIP) approach. In synchronized cultures, MCM complexes associate preferentially with DNA in the intergenic initiation zone early in S-phase during the time that replication initiates. However, significant amounts of MCMs were also detected over the two genes, in agreement with recent observations that the MCM complex co-purifies with RNA polymerase II. As cells progress through S-phase, the MCMs redistribute throughout the DHFR domain, suggesting a dynamic interaction with DNA. In asynchronous cultures, in which replication forks should be found at any position in the genome, MCM proteins were distributed relatively evenly throughout the DHFR locus. Altogether, these data are consistent with studies in yeast showing that MCM subunits localize to origins during initiation and then migrate outward with the replication forks. This constitutes the first evidence that mammalian MCM complexes perform a critical role during the initiation and elongation phases of replication at the DHFR origin in hamster cells.

Although much is known about the mechanism of initiation at the origins of mammalian viruses, little is known about these processes at mammalian chromosomal origins of replication. In viral, yeast, and bacterial replicons, initiation is confined to genetically defined replicator sequences, which direct the loading of initiation factors followed by localized melting of adjacent DNA sequences (reviewed in Ref. 1). This interaction allows access to the origin by primases, polymerases, and other factors involved in the elongation steps of replication. In these relatively simple systems, the term origin is often used to refer to both the replicator (initiation protein binding site) and the local site(s) where nascent strand synthesis begins.

Considerable evidence suggests that initiation at mammalian origins is much more complex. A case in point is the origin in the Chinese hamster DHFR1 domain, which lies in the spacer region between the DHFR and 2BE2121 genes (Fig. 1) (2–4) and which has been analyzed by almost all of the available origin mapping techniques for localizing nascent strand initiation sites (reviewed in Refs. 5 and 6). It was expected that start sites would lie close to replicator sequences that bind to proteins required for initiation. However, these mapping studies have shown that replication can initiate at any one of a very large number of potential sites distributed throughout the 55-kb spacer (4, 7, 8), with at least three subregions (termed ori-β, ori-β’, and ori-γ) being preferred (3, 9, 10). Thus, the ori-β, ori-β’, and ori-γ regions (and possibly others) could correspond to classic genetic replicators. Alternatively, the intergenic region may contain a much larger number of degenerate replicators, with initiation depending more critically on the local environment (e.g. chromatin architecture, transcriptional activity, proximity to matrix attachment regions, etc.).

In the budding yeast, Saccharomyces cerevisiae, chromosomal replicators were identified as autonomously replicating sequence elements, and are recognized by a six-membered protein complex called ORC (for origin recognition complex; reviewed in Refs. 11 and 12). ORC binds to origins throughout the cell cycle and helps to recruit other initiation factors in a stepwise manner (11, 13–16). During G1 phase, the Cdc6 protein is synthesized and localized to origins via interaction with ORC (11, 14, 15, 17). Recruitment of Cdc6, in turn, facilitates recruitment of the mini-chromosome maintenance (MCM) complex (11, 13–19). The assemblage of ORC, Cdc6, and the MCM subunits is collectively called the pre-replication complex, or pre-RC (11, 14–17, 20, 21). Loss-of-function mutations in any of the ORC, MCM, or cdc6 genes in yeast impairs replication and plasmid stability, and reduces the level of initiation occurring
MCMs at a Mammalian Origin

FIG. 1. Organization of the amplified DHFR domain in the CHOC400 genome. The 240-kb DHFR amplicons are arranged in tandem arrays as stable, homogeneously staining regions in CHO 400 cells (58). The map shows only the 120-kb region encompassing the DHFR and 2BE2121 genes and the intergenic spacer. Initiation of replication is confined to the 55-kb spacer. The gray oval corresponds to the bidirectional promoter used by these two genes. Matrix attachment regions (M) are indicated with black squares. Shown below the functional map is the series of cosmids used on dot-blots in the ChIP assays in this report.

at individual origins, suggesting important roles for each of these factors in the initiation reaction (reviewed in Ref. 11).

Although mammalian origins are clearly more complex than those of yeast, homologues of many of the proteins involved in initiation in yeast have been identified in metazoans. Counterparts of all six ORC subunits have been identified in fruit flies (22, 23), several subunits have been found in humans (24–27), two ORC subunits have been identified in frogs (16, 28) and hamsters (29), and the cdc6 gene has been cloned from frogs (15) and humans (30, 31). In addition, all six subunits of the human and Xenopus MCM complex have been identified (32, 33). Considerable biochemical evidence suggests that these proteins are necessary for metazoan replication. For example, microinjecting mammalian cells with antibodies to Cdc6, MCM2, or MCM3 blocks DNA replication (34–37), as does immunodepletion of Cdc6, ORC, or MCM3 subunits from Xenopus extracts (13, 15, 16). Altogether, these data suggest that at least some of the mechanisms involved in identifying and preparing mammalian origins for initiation are likely to be conserved among eukaryotes.

Therefore, it is likely that mammalian cells rely, at least in part, on the formation and proper regulation of pre-RC complexes at or near chromosomal origins of replication, and that yeast homologues of ORC, Cdc6, and MCM proteins perform one or more roles in this process. However, it has not yet been shown that any of these proteins functions in mammalian cells in a manner consistent with their suggested roles in initiation. While there is still no functional assay for ORC and Cdc6 in yeast or any other system, some progress has been made in understanding MCM function during initiation and elongation. For example, recent biochemical evidence suggests that the MCM complex may possess helicase activity (38–42). In addition, in vivo cross-linking studies in yeast have shown that the MCM complex binds to origins of replication at the beginning of S-phase, but then migrates with the replication fork after initiation (14, 40), consistent with a functional role as a helicase.

In this study, we have asked whether MCM subunits are localized preferentially to the DHFR origin region in vivo and whether it is possible to detect any alterations in MCM-chromosome interactions and dynamics that may suggest a functional role for MCMs in replication initiation. We have treated CHO cells with formaldehyde to fix initiation proteins on, or adjacent to, regions of the chromosome where the proteins were situated in vivo. Using this chromatin immunoprecipitation (ChIP) approach, we find that mammalian MCM subunits are preferentially associated with the DHFR origin during the G1/S transition, but become distributed throughout the origin and flanking genes as cells proceed out of S-phase and lose synchrony. These data are consistent with the hypothesis that MCMs localize to this origin during initiation at the beginning of S-phase, and then proceed away from origins with the replication forks.

 MATERIALS AND METHODS

Cell Culture and Synchronization—Chinese hamster CHO 400 cells and human HeLa cells were maintained as monolayers in minimal essential medium supplemented with 10% Fetal Clone II (HyClone) at 37 °C in a humidified atmosphere. CHO 400 cells were synchronized in G1 by starving for isoleucine for 48 h and releasing into complete minimal essential medium containing 400 μM mimosine (Aldrich Chemical Co., Milwaukee, WI) for 12 h (8). Where appropriate, mimosine-containing medium was replaced with drug-free complete medium to allow entry into the S period. Synchronization was verified by analysis of DNA content on a flow cytometer (data not shown).

Western and Immunoprecipitation-Western Analyses—Approximately 5 × 10⁶ CHO 400 (43) or HeLa cells were washed with phosphate-buffered saline (PBS; 150 mM NaCl, 5 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4) and scraped off the plates into cold PBS. Cells were lysed in RIPA buffer for Western analysis (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium deoxycholate, 50 mM NaF, 75 μg of phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitor units of aprotinin), or in 750 μL of TNT for IP-Western analyses (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 75 μg of phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitor units of aprotinin), after which the lysates were sonicated. For Western blots, total protein (50 μg) was separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Membranes were probed according to the recommendations of the supplier. For immunoprecipitation-Western blots, lysates were precleared with protein A-agarose beads (Sigma) and incubated overnight at 4 °C in the presence of the appropriate anti-MCM antibody. Immune complexes were precipitated with protein A-agarose beads and were washed three times with TNT. Immunoprecipitated proteins were separated on 10% SDS-PAGE gels and transferred to membranes as described above. Membranes were probed with 1:2,000 dilutions of the appropriate polyclonal anti-MCM or anti-MEK-1 (Santa Cruz Biotechnology, sc-219) antibodies, and were washed and incubated with a secondary anti-rabbit immunoglobulin-G (IgG) conjugated with horseradish peroxidase (Pierce). Membranes were washed and subjected to enhanced chemiluminescence as described by the manufacturer (Amersham Pharmacia Biotech).

Cesium Chloride Time Course Assay—Approximately 1 × 10⁷ asynchronous CHOC400 cells were collected per cross-linking time duration sample. Cells were washed once with 37 °C PBS and fixed for the indicated times in serum-free medium containing 1% formaldehyde (or no fixative) at room temperature. Cells were washed with cold PBS three times on the plates, scraped into centrifuge tubes, and washed twice again with cold PBS. Pellets were resuspended in 5 ml of RSB (3 mM MgCl₂, 10 mM Tris, pH 8, 10 mM Na bisulfite, pH 8), and allowed to swell for 15 min on ice. Cells were Dounce-homogenized 15 times and centrifuged 10 min at 1100 × g. Cell debris was washed twice with RSB once with SNSB (1 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 0.5% Nonidet P-40, 10 mM Na Bisulfite, pH 8) and pelleted. Pellets were resuspended in 2.25 ml of NSB, 100 mM NaCl and combined with 250 μl of 20% Sarkosyl. Samples were loaded onto a cesium chloride step gradient containing 3 ml of 1.7 g/ml CsCl, 5.5 ml of 1.5 g/ml CsCl, and 3 ml of 1.3 g/ml CsCl. Protein-DNA cross-linked material was separated from non-cross-linked material in a Beckman Ti41 ultracentrifuge rotor at 37,000 RPM at 20 °C for 24 h. The protein-genomic DNA cross-linked products migrated to a density of 1.35 to 1.45 g/ml, depending on cross-linking duration, and were removed with a sterile micro-tip and dialyzed against three changes of 700 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). To obtain proteins for Western analysis, 700 μl of each sample was mixed with 700 μl of methanol and 175 μl of chloro-
form. The samples were vortexed and spun at full speed for 5 min at room temperature in a microcentrifuge. The interface and lower fraction containing protein material were isolated by discarding the upper aqueous phase and 220 μl of methanol was added. The samples were vortexed and spun for 5 min at room temperature. The protein pellet was dried and then resuspended in 100–200 μl of Laemmli loading solution such that the final A$_{260}$ of each sample was equal. Ten microliters of each sample were loaded onto a 10% SDS-PAGE gel and analyzed by Western blotting as described above.

**In Vivo Cross-linking and Hybridization—CHiP assays were performed essentially as described elsewhere (44). Briefly, 3 × 10^7 CHO-C400 cells per sample in 15-cm plates were cross-linked in situ with 10 μl of 1% formaldehyde in serum-free minimal essential medium at room temperature for 5 min. After washing, scraping into Eppendorf tubes, and additional washing with PBS, cell pellets were suspended and incubated once in 1.5 ml of Solution I (10 mM HEPES, pH 7.5, 0.5 mM EGTA, 10 mM EDTA, 0.25% Triton X-100) for 10 min at 4°C, and once in 1.5 ml of Solution II (10 mM HEPES, pH 7.5, 0.5 mM EGTA, 1 mM EDTA, 0.2 mM NaCl) for 10 min at 4°C. Cell pellets were resuspended in RIPA buffer and sonicated to reduce the average DNA fragment to 500–1000 bp in length (verified on agarose gels; data not shown). Cell extracts (1 ml) were precleared with protein A-agarose beads. After antigen-DNA complex precipitation with protein A-agarose beads. After antigen-DNA complexes were eluted from the protein A beads in elution buffer (0.1 M NaHCO$_3$, 1% SDS), they were treated with 250 μg/ml proteinase K for 5 h at 45°C, and cross-links were reversed by incubation at 65°C for 3 h. DNA was extracted with an equal volume of 1:1 phenol/chloroform and an equal volume of chloroform, followed by precipitation with 2 volumes of cold ethanol.

Isolated genomic DNA obtained from equivalent cell numbers in the CHiP assays was labeled by random priming with ^32P (45). The labeled DNA was used to probe Hybond N° dot-blot membranes (Amersham Bioscience, Inc.) that had been loaded with five cosmids covering the DHFR intergenic region and the two adjacent genes (Fig. 1), or with pGEM7 plasmid (1 μg/dot in duplicate; see Fig. 3). Hybridizations were performed as described (46) in the presence of 300 μg/ml of sheared CHO genomic DNA as a source of competitor repetitive DNA sequences (47). Membranes were washed, exposed to x-ray film overnight (for archival purposes), exposed to phosphor screens, and analyzed with a Molecular Dynamics Phosphorimage and ImageQuant software. The raw data were corrected as follows: hybridization signals from anti-MCM ChIP samples were reduced by the amount of raw signal from the control sample from an equivalent number of cells (control samples were non-immune rabbit IgG ChIP sample or Psf-1 preimmune ChIP sample, as appropriate). The resulting values were then normalized to the hybridization signals obtained in an independent probing of an identical cosmid dot-blot with labeled total genomic DNA from CHO-C400 cells, to control for hybridization differences among the cosmid sequences. In no experiments did radiolabeled DNA from any ChIP assay hybridize to pGEM7 DNA.

**RESULTS**

**MCM Subunits in Hamster Cells Are Part of a Multisubunit Complex**—To investigate the interactions of MCM proteins with DHFR origin DNA in hamster cells, we utilized rabbit polyclonal antibodies generated by the Knippers laboratory (Konstanz, Germany) against each of the six full-length human MCM 2–7 subunits. With the exception of the anti-MCM6 preparation, which was whole antisera, antibodies were affinity-purified against the appropriate antigen. Each of these monospecific, polyclonal antibody preparations has been found to specifically recognize the appropriate human MCM subunit homologue using whole cell extract Western analyses or immunoprecipitation approaches (33), 2

Previous studies in human systems have shown that purified human MCMs migrate with the following mobilities: MCM2, 120 kDa; MCM3 and 6, 105 kDa; MCM4, 100 kDa; MCM5, 95 kDa; and MCM7, 85 kDa (33). Western analyses showed that each of the anti-MCM antibodies specifically recognizes the predicted human polypeptide as well as the corresponding hamster protein of nearly identical size (Fig. 2A). We next examined the ability of each antibody to immunoprecipitate its cognate MCM subunit, as well as any other members of the MCM complex that might associate with that subunit. Previous studies on human and *Xenopus* have shown that MCM2, MCM3, and MCM5 form one trimeric subcomplex, and that MCM4, MCM6, and MCM7 form a distinct subcomplex (11, 32, 33, 38, 48). The entire six-membered complex can also be demonstrated in *Xenopus* and human extracts (32, 33, 48). Co-immunoprecipitation-Western analyses show that anti-MCM2 precipitates both MCM2 and MCM5 from hamster cell extracts (Fig. 2B). Similarly, anti-MCM5 precipitates MCM5 and MCM3.

We did not observe co-immunoprecipitation of MCM2, MCM3, and MCM5, possibly because the polyclonal antibodies used may interfere with complex formation. Although MCM7 and, more weakly, MCM3 were immunoprecipitated with their respective antibodies, in neither case was co-immunoprecipitation of any other MCM subunit detected. MCM6 antibodies were not tested in IP-Western analyses, and we did not detect immunoprecipitation of any polypeptides with anti-MCM4. These data show that homologues of the human MCM2–7 subunits are present in hamster cells, and also suggest that at least a subset of hamster MCM polypeptides form complexes, as demonstrated in other experimental systems.

**Chromatin Immunoprecipitation Approach**—To determine whether hamster MCMs are associated preferentially with the Chinese hamster DHFR *in vivo*, we used a ChIP approach described by others (Fig. 3) (44, 49–51). Briefly, whole cells are treated *in vivo* with formaldehyde to cross-link chromatin-bound proteins to the DNA, the cross-linked material is isolated and solubilized by sonication, and any DNA associated

2 R. Knippers, unpublished data.
specifically with the protein of interest is isolated by immunoprecipitation with the cognate antiserum. The DNA thus obtained is then characterized to determine the degree to which known cellular sequences were associated with the protein of interest in vivo at the time of the cross-linking step.

To determine optimal cross-linking conditions, cells were incubated with 1% formaldehyde at room temperature for various intervals (Fig. 4). This was followed by separation of the cross-linked chromatin in cesium chloride gradients and isolation of protein-genomic DNA complexes at a density of 1.4 g/ml (the approximate density of 1:1 protein-DNA chromatin products). The proteins isolated from these complexes were then subjected to immunoblotting with appropriate antibodies. Western analyses clearly showed that MCM3 and MCM5 became maximally cross-linked to genomic DNA between 4 and 8 min in 1% formaldehyde at room temperature; under the same conditions, MCM2 and MCM7 were partially cross-linked by 8 min of treatment (Fig. 4). In contrast, mitogen-activated protein kinase kinase (MEK1), which is primarily a cytoplasmic enzyme (although members of the mitogen-activated protein kinase family have been found in the nucleus; reviewed in Ref. 52), did not become maximally cross-linked to genomic DNA until 30 min or more of formaldehyde treatment. Altogether, these data suggested that treatment with 1% formaldehyde at room temperature for 5–6 min would yield optimal results for studying mammalian MCM-DNA interactions in vivo, in agreement with other established protocols, while largely preventing covalent binding of nonspecific proteins to chromatin (14, 44, 50, 51, 53). These data also show that excessive exposure to formaldehyde can lead to nonspecific cross-linking of clearly irrelevant proteins to chromatin.

In several other studies, DNA obtained in ChIP assays is analyzed by polymerase chain reaction with primer sets that recognize relatively circumscribed region(s) of interest (e.g. promoters (44, 50, 51), discrete origins of replication (14, 53)). However, given the large size and more complex nature of the DHFR origin (reviewed in Refs. 5 and 6), we could not predict a priori how the MCM proteins might be distributed in the genome. We therefore opted for a more unbiased approach in which we could determine the distribution of MCMs throughout the 120-kb region encompassing the DHFR locus, which has been previously characterized vis-à-vis origins (5), transcription units (54),3 matrix attachment regions (55), and chromatin structure (56, 57).

In our approach, the immunoprecipitated DNA sequences themselves were radiolabeled and used to probe dot blots of a series of overlapping cosmids encompassing the DHFR and 2BE2121 transcription units and the initiation zone lying between them (~120 kb; Fig. 1). To increase the signal-to-noise ratio as much as possible, the cross-linking procedure was performed on CHOC400 cells, which have amplified one allele of the 240-kb sequence straddling this region ~1,000 times (58). This amplification factor increases the representation of DHFR-specific sequences in the genomic DNA probe to approximately that of a single copy sequence in S. cerevisiae. In addition, sheared genomic DNA from diploid CHO cells was included in the hybridization reactions as a source of repetitive competitor DNA to eliminate interfering irrelevant signals from labeled repetitive genomic DNA that had been immunoprecipitated. Five cosmids were chosen to allow detection of any differences in distribution between the 55-kb intergenic region and the flanking DHFR and 2BE2121 genes, which are ~28 and 30 kb in length, respectively (see map, Fig. 1).

MCM Proteins Are Distributed Widely Throughout the DHFR Domain in Asynchronous Cells—If MCM2 and MCM5 associate with origins (and in this case with the DHFR origin) prior to and/or during initiation and then move outward with the replication forks, one would predict that, in asynchronous cultures, they would be distributed relatively uniformly throughout the entire 110-kb region represented by the five cosmids utilized in this study (see Fig. 1). After cross-linking with formaldehyde and preparation of the sheared, fixed chromatin samples, ChIP analyses were performed using anti-MCM2 and anti-MCM5 (judged to be the most efficacious for immunoprecipitation of the six antisera characterized in Fig. 2). The purified DNA recovered from the immunoprecipitates was labeled with 32P and used to probe dot blots of approximately equimolar quantities of the indicated cosmids. The resulting hybridization signals were normalized as described under “Materials and Methods,” and the normalized signals were plotted on the map of the DHFR locus at the midpoint of each cosmid insert (Fig. 5).

As predicted, when these two MCM proteins were cross-linked to DNA in asynchronous cultures and then precipitated with the cognate antisera, the resulting patterns showed MCM5 to be relatively uniformly distributed throughout the

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3 T.-H. Leu, A. Pemov, and J. L. Hamlin, unpublished observations.
110-kb region, while MCM2, if anything, appeared to be more highly concentrated over the 2BE2121 gene (Fig. 5, A and B). As a control, we examined the distribution of the transcriptional splicing factor, PSF-1 (59), which would be expected to be enriched over transcription units and not over the origin. As shown in Fig. 5C, antiserum to PSF-1 preferentially immunoprecipitates DNA cross-linked to proteins in the DHFR and 2BE2121 genes. However, a significant amount of PSF-1 is also cross-linked to the intergenic region. Possible reasons for this apparent association of PSF-1 with intergenic sequences will be discussed below.

**MCM Proteins Are Preferentially Associated with the DHFR Intergenic Region in CHOC400 Cells Arrested at the G1/S Interface—Studies on the very efficient ARS1 origin in *S. cerevisiae* have shown that MCMs associate with the origin only during the initiation reaction, and then migrate with replication forks during the elongation phase (14). By analogy, we would predict that MCM proteins should co-localize with the intergenic region prior to entry into the S-phase and move out into the neighboring genes as the cells progress through S-phase.

To synchronize CHOC400 cells at the beginning of the S-period, cultures were first arrested in G1 by isoleucine deprivation, followed by release into complete medium containing the replication inhibitor dimethyl moamine for 14 h to allow cells to accumulate at the beginning of S-phase (8). The drug was washed out and replaced with complete medium and cells were allowed to enter and traverse the S-period. Samples were taken for ChIP analysis at the G1/S boundary and 8 h later, by which time most cells are near the end of the S-period or have begun entering G2 phase (8). Cross-linked DNA was recovered from the chromatin immunoprecipitations as described, was labeled with 32P-labeled poly deoxythymidylate tetrafluoride (32P-PdTp), and was used to probe dot blots of approximately equimolar quantities of the indicated cosmids. The normalized hybridization signals are plotted on the map of the DHFR locus at the midpoint of each cosmid insert (Fig. 6).

MCM2 and particularly MCM5 both appear to associate preferentially with the DHFR intergenic region at the beginning of the S period (Fig. 6, G/S time points). After release from the G1/S block, differences in the distribution of MCM2 and MCM5 among cosmids decrease to the point where they are no longer statistically significant (Fig. 6, G/S time points; note that statistical errors are increased for MCM5). ChIP analyses with anti-MCM7 did not yield any significant hybridization signals from cross-linked DNA compared with that from a rabbit IgG negative control ChIP sample (data not shown).

These data suggest that, prior to initiating replication, hamster MCM2 and MCM5 are preferentially recruited to origins. As cells progress through S-phase, MCMs then appear to migrate with the replication fork machinery, producing a more dispersed pattern of hybridization signals over the cosmids in the ChIP analyses (i.e. the differences between the cosmids hybridization signals become less significant).

**DISCUSSION**

Using antibodies to the human proteins, we have been able to show in Western blots that all six MCM subunits are present in Chinese hamster cells. With regard to interactions among the MCMs in hamster cells, we observed a subcomplex consisting of MCM2, -3, and -5. Similar interactions have been demonstrated in humans and *Xenopus* (reviewed in Ref. 11, 33, and 34). However, we were not able to detect co-precipitation of MCM4, -6, and -7 with antibodies to any of the three subunits even though the three have been demonstrated to form a three-membered complex in *Xenopus* and humans (11, 33, 38). Additionally, all six MCMs have been shown to exist as a single large complex in *Xenopus* and humans (32, 33, 48). Since we were not able to demonstrate the latter two complexes here, it is likely that our method of extract preparation differs in subtle ways from those employed in other studies, or that our antisera may disrupt the holocomplex or subcomplexes of MCM proteins.

In the ChIP experiments themselves, we observed preferential binding of MCM5 and, to a lesser extent, MCM2, to the intergenic DHFR origin when cells were arrested at the G1/S boundary (Fig. 6). As cells progressed through the S-period, the levels of MCMs in the two flanking genes appeared to increase, but the relative amount of binding to the intergenic region did not diminish substantially. By analogy to yeast (14), if the DHFR origin was 100% efficient (i.e. fired in every cell cycle), it would be expected to be cleared of MCMs as they progress away from the origin with the replication forks once initiation in the region ceases (~2 h after entry into the S-phase; Ref. 8). However, this origin fires in only 15–20% of the amiplicons in any one S-period, with the consequence that 80–85% of the origins are replicated passively at later times in S-phase by forks from distant active origins (8, 60). Thus, the MCMs should be concentrated over the intergenic region in early S-phase when 15% of them are active, but should become more uniformly distributed when the cells near the end of S-phase, or in an unsynchronized cell population containing cells at all stages of the cycle. These predictions were, by in large, borne...
Fig. 6. ChIP assays on cross-linked material from synchronized CHOC400 cells. CHOC400 cells were synchronized at the G1/S transition (left panels), or released into S-phase for 8 h (right panels), and were subjected to ChIP analysis as described under “Materials and Methods.” Cross-linked genomic DNA was isolated from each time point using anti-MCM2 (Panel A), anti-MCM5 (Panel B), or non-immune rabbit IgG as a negative control (not shown). The isolated DNA was radiolabeled and used to probe dot-blot of the five cosmids shown (also rabbit IgG as a negative control (not shown). The isolated DNA was radiolabeled and used to probe dot-blot of the five cosmids shown (also see Fig. 1) or pGEM7 vector in the presence of 300 µCi/ml cold competitor, high complexity CHO DNA (47). Hybridization signals obtained with control rabbit non-immune IgG ChIP samples were subtracted from the signals obtained with the anti-MCM antibodies. These data were then adjusted for hybridization efficiencies to the five cosmids by normalizing to the hybridization signals obtained with radiolabeled total genomic DNA from CHOC400 cells. In all cases, a 3–5-fold higher level of labeling and hybridization signals was obtained with the specific anti-MCM antibodies compared with that isolated with rabbit IgG control antibodies. The corrected data are plotted plus or minus 1 S.D. obtained from duplicate cosmid dot blots. Note that the relative quantitative signals obtained among the five cosmids in an individual blot can be validated by this means, but the relative quantitative values observed with DNA obtained with different antibodies or time points, and thus used to probe different blots, cannot be directly compared. The reasoning for the latter is that the antibodies could have different efficiencies, and radiolabeling of immunoprecipitated DNA could be more or less efficient between samples/blots. All blots can, however, be compared with each other qualitatively.

out by the ChIP data on synchronized cells presented in Fig. 6. In chromatin isolated from unsynchronized cultures, MCM5 appears to be relatively uniformly distributed throughout the DHFR region, with no apparent preference for the origin region or the genes. That the ChIP assay is giving a reasonably adequate representation of the in vivo situation is bolstered by preferential binding of the splicing factor PSF-1 to the DHFR and 2BE2121 genes in unsynchronized cells (Fig. 5C). However, there was also considerable binding of PSF-1 to cosmids S21 and SE24, which are completely contained within the intergenic region. Possibly, this results from the fact that, like many other genes (61–63), elongating transcription complexes and their associated splicing complexes (64) continue for some distance beyond the polyadenylation sites in the DHFR and 2BE2121 genes.4

Other studies in higher eukaryotic organisms have suggested that, while localized to the nucleus throughout most of the cell cycle, MCM proteins are tightly associated with chromatin only in G1 and early S-phase, and gradually dissociate from chromatin as cells progress into late S-phase and G2 (11, 13, 35, 65–68). However, the data presented here suggest that MCM proteins are intimately associated with chromatin throughout the cycle. To reconcile our results with those from previous studies, it is important to understand how both sets of data were obtained. In previous studies, chromatin was prepared by isolating nuclear pellets from cytoplasmic and soluble fractions in the presence of Triton X-100 or similar detergent (11, 13, 35, 65–68). These detergents are known to disrupt the nuclear envelope and, in addition, lead to solubilization and extraction of nuclear proteins. The solubilized proteins fractionate with the cytoplasmic supernatant, while the insoluble, detergent-resistant protein fractionate with the nuclear pellet, otherwise referred to as the “chromatin pellet” (67–69). However, the formaldehyde cross-linking approach covalently fixes chromatin-associated proteins in situ, thereby preventing any potential detergent-soluble proteins from being released from their in vivo binding sites during cell extract preparation. In light of this difference, our data suggest that at least some MCM proteins are associated with chromatin (and the DHFR region) even during late S-phase and G2, but in a somewhat different biochemical state than in G1 and early S-phase (detergent-sensitive versus -resistant, respectively).

We had originally anticipated that there would be little or no MCM interactions with genomic DNA over the two genes (DHFR and 2BE2121) adjacent to the intergenic origin region. Thus, the genes were expected to serve as negative controls in samples isolated at the G1/S boundary. The significant amounts of MCM2 and MCM5 detected in the genes therefore could represent nonspecific background cross-linking. In fact, the 2-fold differences that we detect between the genes and the intergenic region at the G1/S boundary are similar to results obtained in virtually all published ChIP assays utilizing PCR approaches in yeast (14, 49, 50) and mammalian cell systems (51). In these studies as well, only 2–3-fold differences were observed in histone, transcription factor, or DNA replication factor binding to specific versus nonspecific genomic DNA sequences.

However, there is another interpretation. Recent work from the Bentley laboratory (70) has shown that the MCM complex copurifies with RNA polymerase II and general transcription factors in high molecular weight complexes. This group went on to show that the interaction is specific and occurs through the carboxyl-terminal domain of RNA polymerase II. In addition, another group has shown that at least one subunit of the MCM complex interacts with the retinoblastoma protein in mammalian cells (71). Since the DHFR promoter has E2F-binding sites and is regulated by E2F (reviewed in Ref. 72), one possible corollary may be that MCM proteins interact with E2F-localized retinoblastoma protein at the DHFR promoter. Thus, it is likely that MCM interactions with genomic DNA in the two genes are detected because MCMs do, indeed, associate with these regions at some points during the cell cycle (notably, during the replicative phase).

This possibility suggests a model for how MCMs might be delivered to the origin region to effect initiation at the beginning of S-phase. The MCMs might be loaded onto chromatin at the promoters of the DHFR and/or 2BE2121 genes via interactions with RNA polymerase II and the transcription machinery during G1, when transcription of the genes is activated (72). The MCMs might then be physically transported into the downstream origin by the advancing transcription machinery. An alternative model is that chromatin folding in the DHFR domain brings the promoters and the intergenic region together.

4 A. Pemov and J. L. Hamlin, unpublished observations.
via a looping mechanism, which has been suggested to explain the long-range effects of enhancers on transcription (73). Thus, the high concentration of MCMs detected in the cosmid representing the genes would be localized near the promoters. A corollary of this latter model is that MCMs would be delivered to the intergenic origin without the need for transcription per se. Interestingly, recent work in our laboratory could support either of these two models: we have shown that deletion of the DHFR promoter region completely inactivates the downstream intergenic origin, demonstrating a genetic interdependence on sequences lying at some distance from the origin.5 We are currently testing whether the act of transcription itself is required for this effect.

In summary, the work presented here suggests that MCM proteins are likely to perform a role in the initiation steps of pre-RC formation in the DHFR origin. Our data are consistent with studies in S. cerevisiae, in which it has been shown that MCM subunits migrate with the replication forks along the DNA after initiation has occurred (14). However, we suggest that the details of the initiation reactions in the two species will differ in some ways to accommodate the demands of development and differential gene expression in higher eukaryotic species.

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A Potential Role for Mini-chromosome Maintenance (MCM) Proteins in Initiation at the Dihydrofolate Reductase Replication Origin
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