Abstract. Previous experiments in Xenopus egg extracts identified what appeared to be two independently assembled prereplication complexes (pre-RCs) for DNA replication: the stepwise assembly of ORC, Cdc6, and M cm onto chromatin, and the FFA-1–mediated recruitment of RPA into foci on chromatin. We have investigated whether both of these pre-RCs can be detected in Chinese hamster ovary (CHO) cells. Early- and late-replicating chromosomal domains were pulse-labeled with halogenated nucleotides and prelabeled cells were synchronized at various times during the following G1-phase. The recruitment of M cm2 and RPA to these domains was examined in relation to the formation of a nuclear envelope, specification of the dihydrofolate reductase (DHFR) replication origin and entry into S-phase. M cm2 was loaded gradually and cumulatively onto both early- and late-replicating chromatin from late telophase throughout G1-phase. During S-phase, detectable M cm2 was rapidly excluded from PCNA-containing active replication forks. By contrast, detergent-resistant RPA foci were undetectable until the onset of S-phase, when RPA joined only the earliest-firing replicons. During S-phase, RPA was present with PCNA specifically at active replication forks. Together, our data are consistent with a role for M cm proteins, but not RPA, in the formation of mammalian pre-RCs during early G1-phase.

Key words: pre-RC • Mcm • RPA • cell cycle • DNA replication domains

In the past several years, much progress has been made in the identification of proteins that assemble onto chromatin to mediate the once-per-cell-cycle replication of eukaryotic chromosomes (Gilbert, 1998). Most of these gene products were originally identified in S. cerevisiae and homologues were subsequently found in higher eukaryotes (Dutta and Bell, 1997; Kearsey and Labib, 1998; Tye, 1999). Studies with Xenopus egg extracts have allowed a preliminary biochemical evaluation of the roles of these gene products in replication (Rowles and Blow, 1997). When Xenopus sperm chromatin is incubated in a Xenopus egg extract, two independent complexes of essential replication proteins are assembled before the initiation of replication. Both of these complexes have been termed prereplication complexes or prereplication centers (pre-RCs). The first type of pre-RC to be identified (A dachi and Laemmli, 1992, 1994) contained replication protein A (RPA), a complex of three polypeptides that constitutes the single-stranded DNA-binding protein (SSB) required for the replication of eukaryotic DNA (Wold, 1997). Previous studies in viral replication systems have shown that stabilization of unwound origin DNA by RPA is one of the earliest steps of the initiation reaction (Tsurimoto et al., 1989). The Xenopus laevis RPA homologue (xRPA) was purified as a stable complex of three protein subunits and shown to be essential for replication of sperm chromatin in Xenopus egg extract (Fang and Newport, 1993; A dachi and Laemmli, 1994). xRPA was found to be rapidly organized into discrete foci on sperm chromatin (A dachi and Laemmli, 1992, 1994; Y an and Newport, 1995a) in a process that requires a partially purified activity termed focus forming activity (FFA-1) and one additional cytosolic fraction (Y an and Newport, 1995a).
Mcm2 association with chromatin immediately after exit from mitosis and during the early stages of G1-phase to determine precisely when Mcm's are recruited to chromatin. In addition, it has been suggested that differential loading of Mcm proteins onto early- vs. late-replicating origins might be responsible for the precise temporal order of chromosomal DNA replication (Kearsey and Labib, 1998). This issue had not previously been addressed, due to the lack of appropriate experimental approaches to examine the G1-phase association of proteins with early- vs. late-replicating chromosomal domains.

Studies of RPA in mammalian cells have not produced a clear picture for its role in the preparation of the genome for replication (Wold, 1997). Evidence for a diffuse nuclear distribution of RPA during G1-phase and to subsequently be displaced from replicating chromatin during S-phase (Todorov et al., 1995; K rude et al., 1996), consistent with a role for Mcm proteins in DNA replication, most likely at a step before initiation. However, these studies did not examine the association of Mcm proteins with chromatin immediately after exit from mitosis and during the early stages of G1-phase to determine precisely when Mcm's are recruited to chromatin. In addition, it has been suggested that differential loading of Mcm proteins onto early- vs. late-replicating origins might be responsible for the precise temporal order of chromosomal DNA replication (Kearsey and Labib, 1998). This issue had not previously been addressed, due to the lack of appropriate experimental approaches to examine the G1-phase association of proteins with early- vs. late-replicating chromosomal domains.

Mammalian Mcm's have been shown to be tightly bound to chromatin during the second half of G1-phase and to subsequently be displaced from replicating chromatin during S-phase (Todorov et al., 1995; K rude et al., 1996), consistent with a role for Mcm proteins in DNA replication, most likely at a step before initiation. However, these studies did not examine the association of Mcm proteins with chromatin immediately after exit from mitosis and during the early stages of G1-phase to determine precisely when Mcm's are recruited to chromatin. In addition, it has been suggested that differential loading of Mcm proteins onto early- vs. late-replicating origins might be responsible for the precise temporal order of chromosomal DNA replication (Kearsey and Labib, 1998). This issue had not previously been addressed, due to the lack of appropriate experimental approaches to examine the G1-phase association of proteins with early- vs. late-replicating chromosomal domains.

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Materials and Methods

Cell Culture and Synchronization

CHO C 400 is a CHO cell derivative in which a 243-kb segment of DNA containing the D HFR gene has been amplified ~500-fold by stepwise selection in methotrexate (H amlin et al., 1994). CHO C 400 cells were grown as monolayer cultures in D ME (GIBCO) supplemented with nonessential amino acids and 5% FBS (GIBCO) at 37°C in a 5% CO₂ atmosphere. Homogeneous populations of cells blocked in metaphase (≈95%) were obtained by mechanical shake off after 4-5 h incubation with nocodazole (Calbiochem-Novabiochem) at 50 ng/ml as described (Gilbert et al., 1995). G1-phase populations were prepared by washing mitotic cells with warm medium and plating in free medium (cells were collected at 2 and 6 h to obtain pre-ODP or post-ODP cells, respectively). G1/S-phase cells were prepared by releasing mitotic cells in free medium for 2-6 h, after which aphidicolin (Calbiochem-Novabiochem) was added at 10 μg/ml and the cells were incubated for another 8-12 h. Cells were released from the aphidicolin block by washing with warm PBS and subsequently incubating for 5 min at 37°C in free medium; under these conditions, ~70-95% of the cells entered S-phase. CHO C 400 S-phase cell populations were prepared by further incubation in free medium for 3 h (early S-phase) or 6-8 h (mid-late S-phase).
Labeling of Nascent DNA with Halogenated Nucleotides

CHO C 400 cells, synchronized at the G1/S border as described above, were pulse-labeled with 10 μM 5-chloro-2'-deoxyuridine (ClUd; Sigma Chemical Co.) for 5–20 min (designated earliest-replicating sequences). The ClUd-medium was removed, cells were washed with warm PBS, chased with 200 μM thymidine (Sigma Chemical Co.), and then regrown in fresh medium. The CHO 400 cells in late S-phase (typically 6–8 h after release from aphidicolin block) were pulse-labeled with 10 μM 5-iodo-2'-deoxyuridine (Iu; Sigma Chemical Co.) for 10 min (designated late-replicating sequences), the Iu-medium was removed, the cells were washed with warm PBS and regrown in fresh medium.

Immunofluorescent Microscopy

The differential staining of DNA sites, substituted with ClUd or Iu, was performed according to the protocol described in (A ten et al., 1992), with some modifications. In experiments where only ClUd- or Iu-substituted DNA was detected, cells were washed with PBS, fixed with cold 70% ethanol and stored at 4°C for an indefinite period of time. Immediately before immunostaining, cells were incubated for 30 min at room temperature in 1.5% NH4Cl, then washed and incubated with the primary antibody. Sites of ClUd incorporation were detected using rat anti-bromodeoxyuridine (BrdU) antibody (no. MA 5200; Harlan-Sera Lab) and FITC-conjugated donkey anti–rabbit IgG (no. 715-095-151; Jackson ImmunoResearch Laboratories). Sites of Iu incorporation were detected using mouse anti-BrDu antibody (no. 347580; Becton Dickinson) and FITC-conjugated donkey anti–mouse IgG (no. 715-095-151; Jackson Immunoresearch Laboratories).

A nitrobenzotriazolyl groups were adjusted specifically for every experiment. Incubations with antibodies were carried out in 10 mm Hepes-KOH, pH 7.4, 300 mm sucrose, 100 mm NaCl, 3 mm no cross-reactivity in double-staining experiments. The primary and secondary antibodies specific for RPA, Mcm2, or PCNA antibodies used were Texas Red (TxRed)-conjugated donkey anti–rabbit IgG (no. sc-56; Santa Cruz Biotechnology). The secondary antibodies were normal staining for Mcm2, but complete lack of or simultaneously with the monoclonal anti-PCNA and the polyclonal anti-Mcm2 antibodies resulted in normal staining for Mcm2, but complete lack of or

Cell Fractionation

CHO C 400 cells were trypsinized, washed with cold PBS and CSK buffer, and then resuspended at 2.5 x 10^6 cells/ml in CSK buffer containing 0.5% Triton, 1 mM PM SF, 1 μg/ml each peptide, chymostatin, leupeptin, and aprotinin, 50 mM sodium fluoride and 0.1 mM sodium vanadate. Cell extraction was carried out for 5 min on ice. Identical results were obtained with different types and concentrations of nonionic detergent (0.05–0.5% Triton or NP-40) and different extraction times (1–10 min). Cell lysates were separated into a soluble fraction and a nuclear pellet by centrifugation for 3 min at 1,500 g at 4°C. The pelleted nuclei were washed once with CSK buffer and resuspended in CSK at 2.5 x 10^6 nuclei/ml. To analyze the amount of total nuclear protein, intact nuclei were prepared by cell permeabilization with digitonin as described (Gilbert et al., 1995; Dimitrova and Gilbert, 1998), with some modifications. CHO C 400 cells (5 x 10^6 cells/ml) were incubated for 5 min on ice in transport buffer (TB) containing 70–80 μg/ml digitonin, 1 mM PM SF, 1 μg/ml each peptide, chymostatin, leupeptin, and aprotinin, 50 mM sodium fluoride, and 0.1 mM sodium vanadate. Cytoplasmic proteins were removed by immediate centrifugation (without addition of BSA stop-solution) at 1,500 g for 2 min at 4°C. The intactness of nuclei was verified before they were used further in the experiment. Digitonin-permeabilized nuclei were prepared by raising the concentration of digitonin to 250 μg/ml (Dimitrova and Gilbert, 1998). Nuclear pellets were washed once with cold TB and resuspended in TB at 2.5 x 10^6 nuclei/ml.

Immunoblotting

Proteins were separated by electrophoresis in SDS-polyacrylamide gels as described (Laemmli, 1970) and electroblotted to nylon membranes (Immobilon, Millipore) using a semi-dry system (Bio-Rad Transblot SD). The membranes were blocked for 1 h in 1% nonfat dry milk in TBS-T buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.05% Tween-20) and probed with the respective primary antibodies, followed by horseradish peroxidase-conjugated goat anti–rabbit (for M cm2 and RPA; no. A-6154; Sigma Chemical Co.) or anti–mouse (for PCNA; no. A-4416; Sigma Chemical Co.) IgG. A nitrobenzotriazolyl group was detected by enhanced chemiluminescence system (ECL; Amersham).

Mapping Replication Origins by the Early Labeled Fragment Hybridization Assay

Specificity of initiation in the DHFR locus in pre- and post-ODP CHOC 400 nuclei was determined by the early labeled fragment hybridization (ELHF) assay as described (Dimitrova and Gilbert, 1998; Gilbert et al., 1995). In brief, ~5 x 10^4 intact nuclei were incubated for 90 min in a X-enzyme egg extract supplemented with 100 μg/ml aphidicolin. Nuclei were then washed free of aphidicolin and the earliest-replicating nascent DNA chromatids were collected in late S-phase and immediately after the protein antigen preceded detection of the halogenated nucleotides. The primary and secondary antibodies specific for RPA, M cm2, or PCNA were fixed in place with 4% formaldehyde for 20 min at room temperature, the cells were treated for 5 min with 0.5% NP-40 in PBS, DNA was depurinated with HCl and the coverslips were subsequently washed with PBS/Tween and incubated with the anti-ClUd or anti-Iu antibodies as described above. Coverslips were mounted in Vectashield (Vector Laboratories).

Conventional epifluorescence microscopy was performed with a Nikon Labophot-2 microscope equipped with a Nikon PlanApo 20X Objective, a dual FITC/R hordamine (Mg erge images) and single FITC and TxrRed fluorescence filters. Photographs were taken on Kodak Ektachrome P1600 films, scanned with a Nikon Coolscan device and assembled in a Power Macintosh A pple G3 computers using A dobe Photoshop 5.0.2 and Claris Draw 1.0x4 software. In Figs. 2C and 4, dual-color confocal laser scanning microscopy was performed with a MRC 1024 ES system (Bio-Rad Laboratories) equipped with a Nikon Eclipse E600 microscope. A Nikon PlanApo 60X Objective was used, and a Krypon/Ar ion laser to excite FITC and TxrRed (at 488 and 568 nm, respectively). Optical sections of 121 pixels x 121 pixels x 8 bit/pixel were collected through the nuclei at 0.5–μm intervals (with Kalman averaging of 6 images). The fluorescence signals from the two fluorochromes were recorded sequentially. Images were processed using LaserSharp software and assembled in A pple G3 computer using A dobe Photoshop 5.0.2 and Claris Draw 1.0x4 software.

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the corresponding values for parallel hybridizations with labeled replication intermediates from exponentially growing cells.

Results

Differential Tagging of Earliest-Replicating and Late-Replicating DNA Domains with Halogenated Nucleotides

In principle, a pre-RC protein could either associate with all replicons during G1-phase or it might associate only with the very first replicons preparing to initiate S-phase. We wanted to design a protocol that could distinguish these two possibilities. In mammalian cells, several replication proteins (DNA polymerase α, cyclinA/cdk2, DNA methyltransferase, DNA ligase I, and PCNA) have been shown to be present at sites of DNA synthesis by simultaneous indirect immunofluorescent labeling of protein antigens and BrdU-substituted nascent DNA (Madsen and Celis, 1985; Kill et al., 1991; Leonhardt et al., 1992; Cardoso et al., 1993; Hozak et al., 1993; Montecucco et al., 1995). However, by design this approach cannot detect the assembly of pre-RCs because it requires that DNA synthesis be already ongoing in order to label sites on DNA. We reasoned that if we could tag early- and/or late-replicating DNA sequences in one cell cycle, then we could follow the association of various proteins with the prelabeled DNA sites during G1-phase of the subsequent cell cycle.

To accomplish this, we took advantage of the ability of specific antibodies to distinguish segments of DNA substituted with iodinated or chlorinated nucleotide analogues (Bakker et al., 1991; A ten et al., 1992) to develop a method of differentially tagging the earliest- and the later-replicating DNA (Fig. 1). CHOC 400 cells were synchronized at the G1/S border by mitotic selection and subsequent incubation in the presence of aphidicolin. Aphidicolin inhibits the processive elongation of nascent DNA strands but does not prevent the initiation of replication and the formation of short (100–500 bp) primers (Netanel and Kaufmann, 1990). Thus, cells accumulate with replication forks arrested close to their sites of initiation. These cells were then released into S-phase by removal of aphidicolin and the very earliest-replicating chromosomal domains were briefly labeled with CldU, followed by a chase period of several hours. Late-replicating DNA was then labeled with IdU, the cells were subsequently synchronized in metaphase by mitotic shake off and released into G1-phase.

It has been shown that the distribution of replication sites in the nucleus follows a defined spatio-temporal program during S-phase, which is typical for each cell type (Nakayasu and Berezney, 1989; Manders et al., 1992; O’Keefe et al., 1992). Examples of these patterns in CHOC 400 cells (a derivative of CHO cells in which the DHFR locus has been amplified 500-fold) are shown in Fig. 1. Each of the fluorescent foci consists of a cluster of replicons that are synchronously replicated within the span of ~60 min (Manders et al., 1996; Ma et al., 1998; Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication). Early replication patterns (visualized as a few tens to a few hundred small fluorescent foci scattered throughout the nuclear interior) persist for the first 5–6 h of a 10–12-h S-phase and consist of multiple sets of replicon clusters. With the protocol shown in Fig. 1, only the very earliest subset of these replicon clusters is labeled with CldU. Since cells do not traverse S-phase in perfect synchrony, the late S-phase IdU-label highlights all three late spatio-temporal replication patterns, providing a convenient means to visualize the entire spectrum of late-replicating domains within the same cell preparations. We have demonstrated that each of these spatio-temporal patterns of labeled replicon clusters persists throughout interphase and is reproduced in the subsequent cell cycle within 2 h after metaphase. Furthermore, we have shown that the earliest subset of clusters tagged with CldU are reproducibly activated at the onset of subsequent S-phases (Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication). Based on this evidence, we conclude that it is feasible to compare the assembly of pre-RCs onto the earliest- vs. later-replicating chromatin by following the behavior of the CldU-tagged and IdU-tagged DNA sites during G1-phase of the subsequent cell cycle.

Mcm2 Binds Simultaneously to Both Early- and Late-replicating Chromatin upon Exit from Mitosis

We examined the localization of chromatin-bound Mcm2 relative to earliest- and late-replicating chromatin in CHOC 400 cell populations prelabeled as described above and released in the subsequent cell cycle for different periods of time. Cells were first extracted with Triton X-100 to remove soluble nuclear proteins, then fixed and stained with a polyclonal antibody specific for Mcm2 and a monoclonal antibody specific for either CldU or IdU. We ob-
Mcm2 Is Loaded Gradually and Cumulatively onto Chromatin throughout G1-Phase

Although there was no qualitative change in the binding of Mcm2 to chromatin during G1-phase, we noticed that the...
Figure 2.
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More effective at removing soluble Mcm2 than digitonin (Pers, 1997). Triton extraction of chromatin was slightly more quantitative than digitonin, which was consistent with results obtained in HeLa cells (Todorov et al., 1995), Chinese hamster M cm2 displays an apparent molecular mass of ~120 kD (Fig. 5 C) and can be resolved as a doublet in the detergent-soluble fraction and as a predominantly single band of lower mobility in the chromatin-bound fraction (Fig. 5 D).

Previous studies of the cell cycle localization of Mcm were not able to evaluate the association of M cm proteins with chromatin during late mitosis and early G1-phase. These experiments were either done with poorly synchronized cells or failed to extract soluble Mcm proteins from the nucleus. Careful inspection of the time between metaphase and early G1-phase revealed that M cm2 first bound to chromatin within 40–60 min after release from nocodazole (Fig. 5 A), coincident with the formation of an intact nuclear envelope in telophase. The percentage of cells in different stages of mitosis was determined by staining aliquots of these cells with DAPI and microscopic observation of mitotic figures (Fig. 5 A). The kinetics of formation of an intact nuclear envelope could be further inferred from the retention of M cm2 in digitonin-permeabilized cells (Fig. 5 A and B, dig70). 20 min after release from nocodazole block, only metaphase and anaphase cells were observed, and no cells had completed the formation of an intact nuclear envelope. Immunofluorescent staining of M cm2 proteins in Triton-extracted metaphase and anaphase cells did not detect any M cm2 bound to chromosomes at these stages of mitosis (Fig. 5 E). The appearance of chromatin-bound M cm2 (corresponding to the bands in the Triton and dig250 lanes in Fig. 5 A) at 40 min after release from nocodazole block was coincident with the formation of an intact nuclear envelope (Fig. 5 A, dig70) in a fraction of the cells and the appearance of telophase cells in the cell population. Immunofluorescent staining revealed that M cm2 was undetectable in Triton-treated early-telophase nuclei and weakly detectable in the late-telophase nuclei (Fig. 5 E). We conclude that M cm2 proteins start to associate with chromatin as soon as an intact nucleus is formed.

Also evident from the immunoblots displayed in Fig. 5 B is an increase in the amount of chromatin-bound M cm2 throughout G1-phase, peaking at the start of S-phase. To determine what percentage of the total M cm2 was bound to chromatin, serial dilutions of the supernatant, obtained after centrifugation of Triton-washed nuclei, were subjected to immunoblotting in parallel with samples of the pellet of chromatin (not shown). Results (Fig. 5 B) revealed that, 90 min after metaphase (the pre-O DP stage of G1-phase), only 20% of the total M cm protein was bound to chromatin. This amount increased to 30% at 6 h after metaphase (post-O DP), reached a peak of 45% at the onset of S-phase (Fig. 5 B: G1/S), and then gradually decreased during S-phase progression. Thus, the association of M cm2 with chromatin does not occur as a single defined step at the end of mitosis; instead, there is a continuous loading of M cm2 onto chromatin throughout G1-phase.

In parallel control experiments, the same protein preparations were subjected to immunoblotting with the antibody against PCNA, which was detected as a single band intensity of anti-M cm2 staining was generally brighter in later stages of G1-phase. In particular, confocal analysis indicated that the colocalization of M cm2 signal (amount of yellow color) with both early- and late-replicating domains increased from early G1 to late G1 phase. To obtain a more quantitative estimate of the amount of M cm2 bound to chromatin at different times during G1-phase, immunoblotting experiments were performed with either whole cell extracts, intact nuclei, permeabilized nuclei or detergent-extracted nuclei. Results (Fig. 5, A and B) revealed that the total amount of M cm2 per cell did not vary significantly throughout the cell cycle and remained exclusively nuclear during interphase. However, the fraction bound to chromatin was regulated during the cell cycle, consistent with previously reported results with HeLa cells (Todorov et al., 1995; Krude et al., 1996; Richter and Kippers, 1997). Triton extraction of chromatin was slightly more effective at removing soluble M cm2 than digitonin permeabilization of nuclei. Varying the concentration of Triton (from 0.05 to 0.5%) or the length of exposure to Triton (1–10 min) did not remove more M cm2, suggesting that this fraction is tightly associated with chromatin. Consistent with results obtained in HeLa cells (Todorov et al., 1995), Chinese hamster M cm2 displays an apparent molecular mass of ~120 kD (Fig. 5 C) and can be resolved as a doublet in the detergent-soluble fraction and as a predominantly single band of lower mobility in the chromatin-bound fraction (Fig. 5 D).
with an apparent molecular mass of 35 kD (Fig. 5 C). As expected, PCNA was detected in the nucleus throughout the cell cycle. It was absent from insoluble nuclear structures during G1-phase, first associated with nuclear components at the G1/S-phase transition and a fraction remained nuclear bound throughout S-phase (Fig. 5 F). Whereas the maximal Mcm2 bound was observed at the G1/S boundary within aphidicolin-blocked cells, the maximal amount of PCNA bound was observed after release from the aphidicolin block, in early S-phase.

**RPA Associates Specifically with Earliest-Replicating Genomic Sequences at the G1/S Border and Redistributes to Sites of Ongoing DNA Synthesis during S-Phase**

Previous studies of RPA in mammalian cells have detected discrete nuclear RPA sites in non-S-phase HeLa cells (Kruke, 1995) or presented direct evidence for a diffuse nuclear distribution of RPA during G1-phase in human cells (Brenot-Bosc et al., 1995; Murti et al., 1996). These results suggested that mammalian RPA may form pre-RCs similar to those observed in Xenopus egg extract and encouraged us to determine exactly when they form in relation to Mcm binding and the ODP. Since the optimal technical protocol for immunostaining of RPA (Dimitrova, D.S., and D.M. Gilbert, manuscript in preparation) was the same as for Mcm2, we were able to use the same populations of synchronized cells shown in Fig. 2, A and B, allowing a direct comparison of the cell cycle behavior of these two proteins. Simultaneous staining of 2 h or 6 h G1-phase nuclei with the anti-RPA antibody and either anti-CldU, or anti-IdU antibody, revealed that RPA was completely absent from G1-phase chromatin (Fig. 6, G1-phase). RPA bound to chromatin at the onset of S-phase (Fig. 6, 5 min in S-phase), when it formed discrete foci that colocalized with sites of early-, but not late-replicating chromatin. Late in S-phase (Fig. 6, 8 h), RPA continued to exhibit punctate distribution but at that time it associated exclusively with late-replicating chromatin domains. Contrary to previous reports (Yan and Newport, 1995a; Murti et al., 1996), RPA foci were not detected in G2-phase nuclei (not shown).

To confirm that there was not a chromatin-bound fraction of RPA in G1-phase nuclei that is not detectable by immunofluorescence, we performed immunoblotting experiments with aliquots of the same protein extracts from synchronized cells shown in Fig. 5. Using the same polyclonal antibody used in Fig. 6, Chinese hamster proteins of 14- (not visible), 30-, and 70-kD apparent molecular masses were detected (Fig. 7 A). Results with synchronized cells (Fig. 7 B) revealed that, as with PCNA, no RPA was detected associated with chromatin during G1-
nuclear Mcm2 proteins. The relative amounts of chromatin-bound Mcm2 proteins at each time point analyzed were estimated by comparing serial dilutions of the soluble fractions run in parallel to an aliquot of the Triton-resistant fraction and are indicated on the right. (C) Western blots of total CHO C400 cellular protein extract probed with anti-Mcm2 or anti-PCNA antibodies. Positions of molecular mass standards (indicated in kD) are marked on the left of each blot. (D) Hamster Mcm2, like human BM28/Mcm2 (Todorov et al., 1995), exists as different isoforms. A lighter exposure of immunoblots probed with Mcm2-specific antibodies reveals that Mcm2 proteins can be resolved as a doublet of bands (marked with stars) in the soluble protein fraction. Only a slowly moving form is detected in the nuclear pellet fraction. (E) Immunofluorescent analysis of Mcm2 chromatin-binding in Triton-extracted CHO C400 cells at different stages of mitosis. A synchronous cells grown on coverslips were extracted with 0.5% Triton in CSK buffer for 2 min on ice, fixed with formaldehyde, and immunostained for Mcm2 as in Fig. 2. DNA was stained with DAPI as in A. The arrowheads point to cells in prophase (i) and (iv), metaphase (ii) and (v), and late telophase (iii) and (vi). The arrows point to a cell in late anaphase/early telophase (iii) and (vi). (F) Immunoblot analysis of chromatin-bound PCNA at different times during interphase. The same protein blots used in B were probed with a PCNA-specific antibody. An increase in the total amount of cellular PCNA at the beginning of S-phase is evident, consistent with previously reported data on human PCNA (Morris and Mathews, 1989). The level of chromatin-bound PCNA increased after release into S-phase and maximal amount of insoluble PCNA was detected during early S-phase.
phase. However, detergent-resistant RPA was readily detected in S-phase cells.

The experiments described above demonstrate that RPA is present in a soluble nucleoplasmic form during G1-phase and binds tightly to the earliest-replicating DNA sites at the onset of S-phase. However, these experiments do not distinguish whether RPA binds nuclear components at some detectable time after the ODP but before initiation of replication. To address this, we prepared mitotic CHO 400 cells and released them into fresh medium without aphidicolin. At 7, 8, or 9 h after release, just when the fastest cells in the population start to enter S-phase, we pulse-labeled aliquots of the cells for 1 min with ClDU, then fixed and double-stained with the anti-RPA and anti-ClDU antibodies (Fig. 8 A). We reasoned that, with very brief ClDU-labeling time, if RPA associated with chromatin several minutes or more before the onset of DNA synthesis, we should be able to detect cells that stained positively for RPA, but not for ClDU. Counting the number of ClDU-positive cells indicated that the percentage of S-phase cells was 9% at 7 h, 20% at 8 h, and 31% at 9 h. These numbers are consistent with results obtained by applying longer ClDU pulses. A s expected, in double-labeled cells the RPA and ClDU sites colocalized, verifying that a 1-min ClDU pulse was sufficient to identify nuclei that synthesized DNA. A t each of these time points, ~5% of cells exhibited positive staining only for RPA, but not for ClDU. No RPA-negative, ClDU-positive cells were found at any time point. Similar results were obtained by staining aliquots of the same cells with antibodies specific for PCNA and ClDU (Fig. 8 B). Thus, we conclude that RPA functions as a protein of the replication machinery assembling into multiprotein nuclear complexes at the G1/S-phase transition, at or very shortly before initiation of DNA synthesis.

Discussion

The order of assembly of replication proteins onto metazoan replication origins is poorly understood due to the paucity of experimental approaches applicable to these systems. In this report, we employ a newly developed indirect immunofluorescence approach to compare the cell cycle-regulated association of early- and late-replicating chromosomal domains with Mcm2 and RPA proteins, two essential replication factors that have been proposed to be a part of mammalian prereplication complexes. We demonstrate that, in CHO fibroblasts, Mcm2 associates with both early- and late-replicating chromatin as soon as nuclear envelopes are assembled in telophase. Subsequently, additional Mcm2 is loaded onto chromatin throughout G1-phase and is maximal at the G1/S border. Detectable Mcm2 is then displaced from replicons shortly after their initiation. By contrast, we found no evidence for stable association of RPA with chromatin during G1-phase. These properties are consistent with Mcm proteins, but not RPA, being a part of mammalian early G1-phase pre-RCs.

Mcm2 Is a Component of the Hamster pre-RCs

Previous studies in human, mouse, and Xenopus cells have concluded that members of the Mcm family of proteins are present in the nucleus throughout interphase but associate with chromatin only during part of the cell cycle, beginning at some undefined point in G1-phase (Todorov et al., 1995; Krude et al., 1996; Richter and Knippers, 1997). Synchrony methods employed in these prior studies were not sufficient to determine whether Mcm proteins were binding during early or late G1-phase and were not able to relate chromatin association of Mcm proteins to specific G1-phase hallmarks, such as the ODP (Wu and Gilbert, 1996). When synchronized in metaphase with a brief nocodazole block, followed by mechanical shake-off of mitotic cells, CHO 400 cells proceed into G1-phase within 60–90 min in a highly synchronous fashion. This allowed us to look more precisely at the timing of the Mcm-chromatin interactions during the mammalian G1-phase. We show here that ~20% of hamster Mcm2 associates with chromatin as cells exit mitosis, several hours before specification of the DHFR replication origin at the ODP. In fact, initial binding of Mcm2 coincides with the assembly of a nuclear envelope, which would allow active transport to concentrate
Mcm proteins in the nuclear compartment. Significantly, our novel indirect immunofluorescence approach for visualization of proteins and tagged DNA sites allowed us to demonstrate for the first time that Mcm2 proteins bind simultaneously to both early- and late-replicating chromatin regions at the very beginning of G1-phase. Quantitative immunoblotting analysis showed that an additional 25% of Mcm2 binds chromatin gradually and cumulatively throughout G1-phase until, at the G1/S border, 45% of Mcm2 is bound, with the remainder present in the soluble nucleosolic fraction. A similar increase in the amount of chromatin-bound Mcm proteins during G1-phase has been documented in S. cerevisiae (Aparicio et al., 1997; Tanaka et al., 1997; Zou and Stillman, 1998). The observation that the initial binding of hamster Mcm2 to chromatin occurs in late telophase is similar to the situation in yeast and Xenopus egg extracts and implicates this protein as a pre-RC component. The fact that this event is upstream of the ODP suggests that the formation of Mcm-containing pre-RCs is not sufficient for the specification of mammalian replication origins. This view is also supported by studies in Xenopus egg extracts, where Orc-Cdc6-Mcm-dependent replication occurs without the use of specific origins (Gilbert, 1998). However, since we found that additional Mcm is continuously loaded onto hamster chromatin throughout G1-phase, it is still formally possible that a critical threshold of loaded Mcm in some way focuses initiation to specific sites. Alternatively, the specification of origins could be a gradual process, with different origins specified at different times in G1-phase corresponding to the binding of Mcm proteins. To date, the ODP has been determined only for the DHFR origin. In eukaryotic nuclei, the activation of replication origins occurs according to a strictly regulated temporal program (Fangman and Brewer, 1992; Diller and Raghuraman, 1994). Although the mechanism that establishes this program has not been elucidated, it has been suggested that replication timing might be influenced by the amount or...
kinetics of Mcm loading onto early- vs. late-replicating chromatin (Kearsey and Labib, 1998). The technique described in this report allowed us to distinguish the association of proteins with early- and late-replicating chromosomal domains during G1-phase. We consistently observed equivalent association of Mcm2 with early- and late-replicating chromatin. Hence, the establishment of a chromosomal domain as early- or late-replicating does not appear to involve quantitative differences in the association of Mcm2 proteins.

Are Mcm Proteins Present at DNA Replication Forks: The Mcm Paradox

In S. cerevisiae chromatin immunoprecipitation experiments have produced direct evidence for the binding of members of the Mcm protein family (Mcm4 and Mcm7) to yeast chromosomal replication origins (Aparicio et al., 1997; Tanaka et al., 1997). One of these studies further showed that shortly after origin firing Mcm4 and Mcm7 proteins dissociate from origins and move with replication forks, along with DNA polymerase ε (Aparicio et al., 1997). This observation raised an apparent paradox regarding the behavior of Mcm proteins (Newton, 1997), since previous reports (and our own unpublished data) have shown that, in Xenopus egg extracts and in cultured mammalian cells, members of the Mcm family do not colocalize with sites of newly synthesized DNA (Kruše, 1995; Todorov et al., 1995; Rahmanowski et al., 1996a). However, these results are difficult to interpret because proteins are localized to their positions at the moment of fixation, whereas sites of newly synthesized DNA were labeled before fixation. In the approach described in this report, proteins can be localized to sites that are actively engaged in replication. Mcm2 was still associated with the earliest-firing replication clusters after initiation (at the aphidicolin-arrested step) but was cleared from those earliest replicating clusters within 5 min after DNA synthesis was allowed to proceed. At this time, these CldU-tagged sequences were actively being replicated and colocalized with the replication fork proteins PCNA and RPA. Since these earliest replication clusters take ~60 min to complete replication (Manders et al., 1996; Ma et al., 1998; Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication), we are forced to conclude that all detectable (by immunofluorescence) mammalian Mcm2 is cleared from replication sites after initiation (see Fig. 2, A and C, 5 min in S-phase) and does not associate with replication forks thereafter. Furthermore, direct inspection of Mcm2 and PCNA revealed a complete lack of colocalization (Fig. 4A). The possibility remains that other members of the Mcm complex, such as Mcm4 (Aparicio et al., 1997), behave differently than Mcm2. However, Tanaka et al. (1997) did not observe association of Mcm4 with S. cerevisiae replication forks using the same technique as (Aparicio et al., 1997). It is also possible that there is a distinct minor population of Mcm proteins that escape immunodetection but are present at replication forks. This would imply the existence of three populations of Mcm proteins: a soluble form (at least half), a chromatin-bound immunodetectable form that is cleared upon initiation, and a replication fork-associated form.

RPA Does Not Form pre-RCs during Early-G1 Phase in Mammalian Nuclei

While Mcm2-binding to chromatin before the ODP implicates it as a component of the CHO pre-RCs, the behavior of RPA is most consistent with its involvement in the initiation and elongation of nascent DNA strands. RPA is a well-characterized complex of three polypeptides that is present at DNA replication origins in both human cells (Adachi and Laemmli, 1994; Yan and Newport, 1995a) and Xenopus egg extracts (Adachi and Laemmli, 1992). In human cells, RPA is necessary for the stably recruitment of DNA polymerase δ to the origin (Adachi and Laemmli, 1994; Yan and Newport, 1995a), and it is also required for the initiation of DNA synthesis (Adachi and Laemmli, 1994; Yan and Newport, 1995b). In Xenopus egg extracts, RPA associates with sperm chromatin before the start of DNA synthesis. We have applied the same immunofluorescent technique employed to examine the association of Mcm2 with chromatin and failed to detect any stable interaction between RPA and chromatin during G1-phase in CHO C40 cells. Hamster RPA assembled into distinct nuclear granules within a few minutes before DNA synthesis and could be detected in association with the earliest-firing replication clusters at the onset of S-phase, similar to PCNA. This coincides with the initial appearance of replicative megacomplexes, termed replication factories, which have been observed to form at the G1/S transition in human fibroblasts (Kil et al., 1991; Hozak et al., 1994) and contain both PCNA and DNA polymerase α. Our results are also consistent with recent reports on different phases of DNA replication. Mt and D.M. Gilbert, manuscript in preparation) whose functional significance for DNA replication remains unclear.

The approach described in this report also allowed us to demonstrate that RPA does not associate with late-replicating chromosomal regions until the late stages of S-phase, at the time when these sequences engage in replication. This behavior parallels that of PCNA and supports a role for RPA in the initiation and elongation steps of replication, not the formation of pre-RCs.

What Are the Xenopus RPA-containing pre-RCs?

Most of our knowledge of the role of RPA in replication has resulted from in vitro studies of SV-40 replication or Xenopus sperm chromatin replicating in Xenopus egg extracts. In the SV-40 system, RPA has been shown to be required for the stabilization of unwound origin DNA during the initiation step (Tsuriel et al., 1989). In Xenopus egg extracts, RPA is essential for replication and forms punctate foci on sperm chromatin that resemble sites of DNA synthesis (A dachi and Laemmli, 1994; Yan and Newport, 1995b). The assembly of RPA foci on chromatin precedes DNA unwinding and the initiation of replication and is independent of cdk2 activity in the extracts, implying that RPA may play a role at an earlier, preinitiation stage of chromosome replication (A dachi and Laemmli, 1994; Yan and Newport, 1995a). Subsequently, it was shown that the formation of prereplicative RPA foci on sperm chromatin is dependent on the presence of another
protein, FFA-1, later shown to be the Xenopus homologue of a human DNA helicase defective in individuals with Werner syndrome (Yan and Newport, 1995b; Yan et al., 1998). RPA-containing pre-RCS do not colocalize with X. elegans MCM proteins (Coue et al., 1996) and are assembled in extracts that have been immunodepleted of Xenopus ORC and CDC6 proteins and cannot form MCM-containing pre-RCS (Coleman et al., 1996). Based on these findings, it has been concluded that two separate and independent assembly pathways are essential for the initiation of replication in eukaryotic cells.

After these studies, it has been assumed that similar prereplicative RPA foci exist in mammalian cells. However, we could find no evidence for their existence and recent studies in S. cerevisiae found no evidence for RPA association with yeast chromatin in G1-phase (Tanaka and Nasmyth, 1998). In fact, it still remains to be demonstrated that FFA-1/RPA foci formed in Xenopus egg extracts are involved in DNA replication. Oddly, RPA foci were found to persist on sperm chromatin even after completion of DNA replication (in G2-phase; Yan and Newport, 1999b).

Most importantly, while it has been shown that RPA foci do not form in FFA-1-depleted extracts, the critical experiment to determine whether FFA-1-depleted extracts could support sperm DNA replication was not performed (Yan et al., 1998). FFA-1 is a homologue of Werner’s helicase which, like RPA, and possibly together with RPA, is involved in DNA repair and recombination (Fry and Loebl, 1998; Brosh et al., 1999; Suzuki et al., 1999). Hence, it remains possible that FFA-1-mediated formation of RPA foci is unrelated to DNA replication and that the role of RPA in DNA replication is restricted to its role as defined in the SV-40 in vitro studies.

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