Mcm2, but Not RPA, Is a Component of the Mammalian Early G1-Phase Prereplication Complex

Daniela S. Dimitrova,* Ivan T. Todorov,‡ Thomas Melendy,§ and David M. Gilbert*

*Department of Biochemistry and Molecular Biology, S.U.N.Y. Health Science Center, Syracuse, New York 13210; ‡Desmos, Inc., San Diego, California 92121; and §Department of Microbiology, S.U.N.Y. at Buffalo, School of Medicine and Biomedical Sciences, Buffalo, New York 14214

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 Mcm 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 Mcm2 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. Mcm2 was loaded gradually and cumulatively onto both early- and late-replicating chromatin from late telophase throughout G1-phase. During S-phase, detectable Mcm2 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 Mcm 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-RC)[1]. The first type of pre-RCs to be identified (Adachi 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 (xlRPA) 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; Adachi and Laemmli, 1994). xlRPA was found to be rapidly organized into discrete foci on sperm chromatin (Adachi and Laemmli, 1992, 1994; Yan and Newport, 1995a) in a process that requires a partially purified activity termed focus forming activity (FFA-1) and one additional cytosolic fraction (Yan and Newport,

Address correspondence to David M. Gilbert, Department of Biochemistry and Molecular Biology, S.U.N.Y. Health Science Center, 750 East Adams Street, Syracuse, NY 13210. Tel.: (315) 464-8723. Fax: (315) 464-8750. E-mail: gilbertd@vax.cs.hscsyr.edu

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CldU, 5-chloro-2′-deoxyuridine; DHFR, dihydrofolate reductase; FFA, focus forming activity; IdU, 5-iodo-2′-deoxyuridine; ODP, origin decision point; ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; pre-RCs, prereplication complexes or centers; RPA, replication protein A; SSB, single-stranded DNA-binding protein; TxRed, Texas Red; xlRPA, *Xenopus laevis* RPA homologue.

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1995b). These RPA foci were shown to form on chromatin before DNA unwinding, and under conditions in which the initiation of replication was prevented. Hence, they have been referred to as prereplication centers (pre-RCs).

Subsequent to the identification of RPA pre-RCs, the *Xenopus* homologues of the *S. cerevisiae* origin recognition complex (ORC), Cdc6, and Mcm2-7 proteins were identified, shown to be essential for replication in *Xenopus* egg extracts, and found to rapidly associate with sperm chromatin introduced into *Xenopus* egg extract (Kubota et al., 1995; Madine et al., 1995; Carpenter et al., 1996; Chong et al., 1995; Coleman et al., 1996). The sequential order of association of these proteins was shown to be ORC, Cdc6, and Mcm, with the binding of each dependent upon the prior association of the upstream proteins (Coleman et al., 1996; Romanowski et al., 1996b). The *S. cerevisiae* counterparts of these proteins were subsequently shown to assemble in this same order onto the specific sequences that function as origins of replication (Aparicio et al., 1997; Tanaka et al., 1997). Since the ORC-Cdc6-Mcm pathway appears to be conserved in eukaryotes, is required for replication, and is completed before the initiation step, this multiprotein complex is also referred to as a prereplication complex (pre-RC). However, the relationship between these pre-RCs and the RPA-containing pre-RCs is unclear.

In mammalian cells, 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; Krude 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-replication 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 suggested that its role as a pre-RC component may be evolutionarily conserved (Brenot-Bosc et al., 1995; Murti et al., 1996). On the other hand, controversial reports on the cell cycle–regulated dissociation of the extremely stable RPA complex or the lack of dynamic localization of some of the RPA subunits to replication foci during S-phase (Cardoso et al., 1993; Murti et al., 1996) have produced considerable confusion. Clearly, elucidating the role of RPA is critical to our understanding of replication origin assembly in mammalian cells.

One approach to study the roles of initiation proteins is to determine their order of assembly during the cell cycle relative to other defined replication hallmarks. Recently, we identified a distinct early G1-phase step at which CHO cell nuclei become committed to initiate replication at specific sites within the dihydrofolate reductase (DHFR) locus, termed the origin decision point (ODP) (Wu and Gilbert, 1996). We reasoned that a detailed examination of the association of Mcm and RPA proteins with chromatin during early G1-phase, particularly in relation to the ODP, would help to clarify whether both of the previously defined pre-RCs exist in mammalian cells and how their assembly relates to the selection of specific origins of replication at the ODP. We applied a newly developed approach (Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication) in which the DNA segments that replicate either early or late in S-phase are differentially tagged and monitored throughout the CHO cell cycle. Here we demonstrate that this approach can be applied to examine the association of proteins with early- and late-replicating chromatin during G1-phase. We found that Mcm2 proteins bind to both early- and late-replicating chromatin very early in G1-phase, before the ODP. Unexpectedly, we observed that the amount of chromatin-associated Mcm2 increased gradually throughout G1-phase. However, no qualitative changes or biphasic transitions in this association were observed at the ODP, suggesting that the simple act of Mcm2 loading onto chromatin is not directly related to the selection of specific origin sites at the DHFR locus. By contrast, RPA associated with earliest-replicating genomic sequences only at the onset of S-phase and dynamically redistributed to sites of ongoing DNA synthesis throughout S-phase, forming punctate foci that closely resembled and colocalized with those formed by the proliferating cell nuclear antigen (PCNA), a well-characterized replication fork protein. We conclude that mammalian Mcm2 association with chromatin is consistent with the formation of a pre-RC complex on chromatin before the specification of replication origins. However, we find no evidence for a role of RPA in the formation of pre-RCs in mammalian nuclei. Instead, our data provide a global and unifying view for the role of RPA in genome replication in simple and complex organisms.

Materials and Methods

Cell Culture and Synchronization

CHOC 400 is a CHO cell derivative in which a 243-kb segment of DNA containing the DHFR gene has been amplified ∼500-fold by stepwise selection in methotrexate (Hamlin et al., 1994). CHOC 400 cells were grown as monolayer cultures in DME (GIBCO) supplemented with nonessential amino acids and 5% FBS (GIBCO) at 37°C in a 5% CO_2 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. CHOC 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

CHOC 400 cells, synchronized at the G1/S border as described above, were pulse-labeled with 10 μM 5-chloro-2′-deoxyuridine (CldU; Sigma Chemical Co.) for 5–20 min (designated earliest-replicating sequences). The CldU-medium was removed, cells were washed with warm PBS, chased with 200 μM thymidine (Sigma Chemical Co.), and then regrown in fresh medium. CHOC 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 (IdU; Sigma Chemical Co.) for 10 min (designated late-replicating sequences), the IdU-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 CldU or IdU, was performed according to the protocol described in (Aten et al., 1992), with some modifications. In experiments where only CldU- or IdU-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 N HCl, then washed and incubated with the primary antibody. Sites of CldU incorporation were detected using rat anti-bromodeoxyuridine (BrdU) antibody (no. MAS250b; Harlan-Sera Lab) and FITC-conjugated donkey anti–rat IgG (no. 712-095-153; Jackson ImmunoResearch Laboratories). Sites of IdU 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). Antibody concentrations were adjusted specifically for every experiment. Incubations with antibodies were carried out in a humidified chamber for 0.5–1 h at room temperature, or for longer time periods at 4°C. Multiple controls, using nonlabeled DNA or omitting the respective primary antibodies, were performed to ensure that there was absolutely no cross-reactivity in double-staining experiments.

For immunostaining of protein antigens, CHOC 400 cells grown on coverslips were washed with cold PBS and cold cytoskeleton buffer (CSK: 10 mM Hepes-KOH, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$) and then extracted for 2 min on ice with 0.5% Triton X-100 (Triton; Sigma Chemical Co.) in CSK buffer supplemented with 1 mM PMSF (Boehringer Mannheim), 1 μg/ml each pepstatin, chymostatin, leupeptin, and aprotinin (Sigma Chemical Co.), 50 mM sodium fluoride and 0.1 mM sodium vanadate. The cells were fixed for 20 min at room temperature with 4% formaldehyde in PBS, washed with PBS, treated for 5 min with 0.5% NP-40 (Boehringer Mannheim) in PBS, and stored in PBS at 4°C. The following primary antibodies were used for detection of protein antigens: (a) affinity-purified rabbit polyclonal anti-human Mcm2 antibody (Todorov et al., 1995); (b) rabbit polyclonal anti-human RPA antibody (Din et al., 1990); and (c) mouse monoclonal anti-human PCNA antibody (PC10 mAb, no. sc-56; Santa Cruz Biotechnology). The secondary antibodies used were Texas Red (TxRed)-conjugated donkey anti–rabbit IgG (no. 711-075-152; Jackson ImmunoResearch Laboratories), FITC-conjugated donkey anti–rabbit IgG (no. 711-095-152; Jackson ImmunoResearch Laboratories) and TxRed-conjugated donkey anti–mouse IgG (no. 715-075-151; Jackson ImmunoResearch Laboratories). The order of addition of the primary antibodies was found to be essential in the Mcm2+PCNA staining experiment in Fig. 4 A. Incubating the cells simultaneously with the monoclonal anti-PCNA and the polyclonal anti-Mcm2 antibodies resulted in normal staining for Mcm2, but complete lack of or very faint staining for PCNA. Hence, the cells were first incubated with the anti-PCNA, followed by the anti-Mcm2 antibodies. This effect was not observed in the RPA+PCNA staining experiment in Fig. 4 B, in which the cells were incubated simultaneously with the monoclonal anti-PCNA and the polyclonal anti-RPA antibodies. All washes after antibody incubations were done with 0.5% Tween-20 (Sigma Chemical Co.) in PBS (PBS/Tween) at room temperature.

In double-staining experiments where proteins were colocalized with sites of CldU- or IdU-substituted DNA, the cells were fixed according to the protocol described for the respective protein and immunolabeling of the protein antigen preceded detection of the halogenated nucleotides. The primary and secondary antibodies specific for RPA, Mcm2, 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-CldU or anti-IdU 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 100× 1.4 NA oil-immersion objective, a dual FITC/Rhodamine (Merge images) and single FITC and TxRed fluorescence filters. Photographs were taken on Kodak Ektachrome P1600 films, scanned with a Nikon Coolscan device and assembled in a Power MacIntosh and Apple G3 computers using Adobe Photoshop 5.0.2 and Claris Draw 1.0v4 software. In Figs. 2 C 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 60× 1.4 NA oil-immersion objective was used, and a Krypton/Argon laser to excite FITC and TxRed (at 488 and 568 nm, respectively). Optical sections of 512 pixels × 512 pixels × 8 bits/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 an Apple G3 computer using Adobe Photoshop 5.0.2 and Claris Draw 1.0v4 software.

Cell Fractionation

CHOC 400 cells were trypsinized, washed with cold PBS and CSK buffer, and then resuspended at 2.5×10^7 cells/ml in CSK buffer containing 0.5% Triton, 1 mM PMSF, 1 μg/ml each pepstatin, 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 2.5×10^7 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. CHOC 400 cells (5×10^6 cells/ml) were incubated for 5 min on ice in transport buffer (TB) containing 70–80 μg/ml digitonin, 1 mM PMSF, 1 μg/ml each pepstatin, chymostatin, leupeptin, and aprotinin, 50 mM sodium fluoride, and 0.1 mM sodium vanadate. Cytosolic 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 2.5×10^7 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 4% 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 Mcm2 and RPA; no. A-6154; Sigma Chemical Co.) or anti–mouse (for PCNA; no. A-4416; Sigma Chemical Co.) IgG. Antibody binding 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 (ELFH) assay as described (Dimitrova and Gilbert, 1998; Gilbert et al., 1995). In brief, $\sim 5 \times 10^5$ intact nuclei were incubated for 90 min in a *Xenopus* egg extract supplemented with 100 μg/ml aphidicolin. Nuclei were then washed free of aphidicolin and the earliest-replicating nascent DNA chains were labeled briefly with α-[^{32}P]dATP (New England Nuclear). 17 unique probes (Gilbert et al., 1995; Lawlis et al., 1996) distributed over a 120-kb region that includes the DHFR ori-β were immobilized on nylon filters (Hybond N+; Amersham) and hybridized to the ^{32}P-labeled early replication intermediates. Relative cpm were obtained by PhosphorImaging analysis (Molecular Dynamics) and adjusted for differences in probe size, deoxyadenine content, and hybridization efficiency by normalizing to

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; Aten 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 (Nethanel 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.

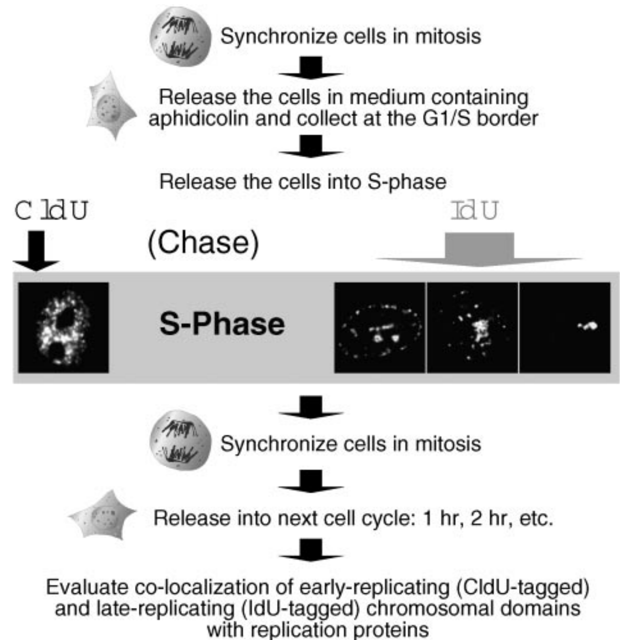


Figure 1. Experimental protocol (see text for details). Representative photographs of the types of replication patterns prevailing in the early (first half, euchromatic) or late (second half, three sequential heterochromatic patterns) stages of the 10–12 h S-phase in CHOC 400 cells are displayed.

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-

served that Mcm2 began to associate with both early- and late-replicating chromatin within Triton-extracted nuclei as soon as the daughter nuclei were formed at the end of mitosis. There was no qualitative difference in the Mcm2 staining pattern between pre-ODP (2-h time point in Fig. 2, A and B) and post-ODP nuclei (6-h time point in Fig. 2, A and B). To verify that these cells were in fact in the pre-ODP and post-ODP stages of G1-phase, intact nuclei prepared from aliquots of the same cell cultures were introduced into *Xenopus* egg extract and the sites of in vitro initiation of replication at the DHFR locus were mapped (Fig. 2 D). These results confirmed that neither the complicated synchronization procedure, nor the tagging of DNA sequences with halogenated nucleotides exerted any deleterious effects on the progression of cells through G1-phase. We conclude that Mcm2 association with chromatin is predominantly upstream of the DHFR ODP and that no qualitative changes in Mcm2 distribution take place at the ODP.

Nearly uniform association of Mcm2 with chromatin continued throughout G1-phase and persisted in aphidicolin-arrested (G1/S) cells. Remarkably, within a few minutes after the release of cells into S-phase, when CldU-tagged sequences are actively replicating, a significant amount of Mcm2 was absent from these earliest-replicating clusters. Since early foci take an average of 60 min to complete replication (Manders et al., 1996; Ma et al., 1998; Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication), Mcm2 is either released from active replicons or the epitope is rapidly masked. Mcm2 proteins were clearly still present on the remaining chromatin, as manifested by the intense staining of the nuclear interior. These results are most consistent with the rapid release of Mcm2 proteins from the very first replicons to fire and the persistence of Mcm2 on the remaining sets of replicon clusters, including the remaining early-firing replicons. By 3 h into S-phase, when more than half of the early replicating foci have completed replication (Dimitrova, D.S., and D.M. Gilbert, manuscript submitted for publication), detectable Mcm2 was cleared from a large fraction of the early-replicating chromatin and the intensity of the immunofluorescent signal in the nuclear interior had significantly decreased. However, Mcm2 proteins remained associated with late-replicating chromatin throughout early S-phase (Fig. 2 B) and were gradually released from these domains at times corresponding to their replication times.

The data described above were obtained with a conventional epifluorescence microscope equipped with a high numerical aperture lens to restrict the image collected to a relatively thin focal plane. To further substantiate that the colocalization of Mcm2 with earliest- and late-replicating chromatin was not due to overlap of independent signals from different focal planes, confocal images were collected using z-sections of 0.5 μm. This was particularly important to confirm colocalization of Mcm2 with the earliest-replicating sequences (which are distributed throughout the interior of the nucleus) and the separation of Mcm2 from those sequences shortly after initiation of replication. Results (Fig. 2 C) confirmed that detectable Mcm2 is released from chromatin domains shortly after they initiate DNA replication.

As a control, aliquots of these same cells were used to colocalize the earliest-replicating DNA domains with PCNA, which functions at the elongation stage of DNA replication and is clearly not a component of the pre-RC. PCNA is present in the nucleus throughout the cell cycle but associates with chromatin only during S-phase, localizing to sites of DNA synthesis that appear as punctate foci within the nucleus (Madsen and Celis, 1985; Bravo and Macdonald-Bravo, 1987; Morris and Mathews, 1989; Kill et al., 1991). Since both PCNA and IdU are detected with mouse monoclonal antibodies, we could not visualize them simultaneously. Instead, we prepared parallel cultures of prelabeled cells, singly pulse-labeled with CldU late in S-phase. Consistent with previous reports (Bravo and Macdonald-Bravo, 1987), indirect immunofluorescent staining showed that, during G1-phase, PCNA was completely removed from CHOC 400 nuclei by extraction with Triton (Fig. 3, G1-phase). PCNA assembled into detergent-resistant replication granules at the onset of S-phase (Fig. 3, 5 min in S-phase), colocalizing to a significant extent with the earliest-replicating chromatin. As cells progressed further into S-phase, the percentage of PCNA foci that colocalized with earliest-replicating chromatin was reduced and PCNA was redistributed to later-firing subsets of euchromatic replicons. However, in contrast to Mcm2 proteins, PCNA was completely excluded from heterochromatin for the first 5–6 h of S-phase (Fig. 3 B). PCNA associated transiently with late-replicating domains exclusively at their respective scheduled replication times (Fig. 3 B, 8 h).

The data in Fig. 2 indicate that Mcm2 is released from the earliest-firing replicons before the completion of their replication. This result appears to be in contradiction to experiments in *S. cerevisiae* that have revealed the presence of Mcm proteins at active replication forks (Aparicio et al., 1997). The fact that PCNA colocalizes with the CldU-labeled sequences suggests that, if Mcm2 was also a replication fork protein, then we should also have observed a significant degree of colocalization of Mcm2 with the CldU-labeled sequences. Nonetheless, to directly examine the presence of Mcm2 at replication forks, we stained aliquots of the same cells shown in Fig. 2 C simultaneously for PCNA and Mcm2 and analyzed them by confocal microscopy (Fig. 4 A). No colocalization of PCNA and Mcm2 was observed at any time during S-phase. We considered the possibility that the monoclonal anti-PCNA and polyclonal rabbit anti-Mcm 2 antibodies might somehow mask each other and prevent the detection of colocalizing signals at replication forks. Hence, we performed control experiments double-staining aliquots of these same samples with monoclonal anti-PCNA and polyclonal rabbit anti-RPA antibodies (RPA is also present at the replication fork, see below). As expected, a high degree of colocalization was observed between PCNA and RPA throughout S-phase (Fig. 4 B). We conclude that there is no immunologically detectable Mcm2 protein at mammalian replication forks.

Mcm2 Is Loaded Gradually and Cumulatively onto Chromatin throughout G1-Phase

Although there was no qualitative change in the binding of Mcm to chromatin during G1-phase, we noticed that the

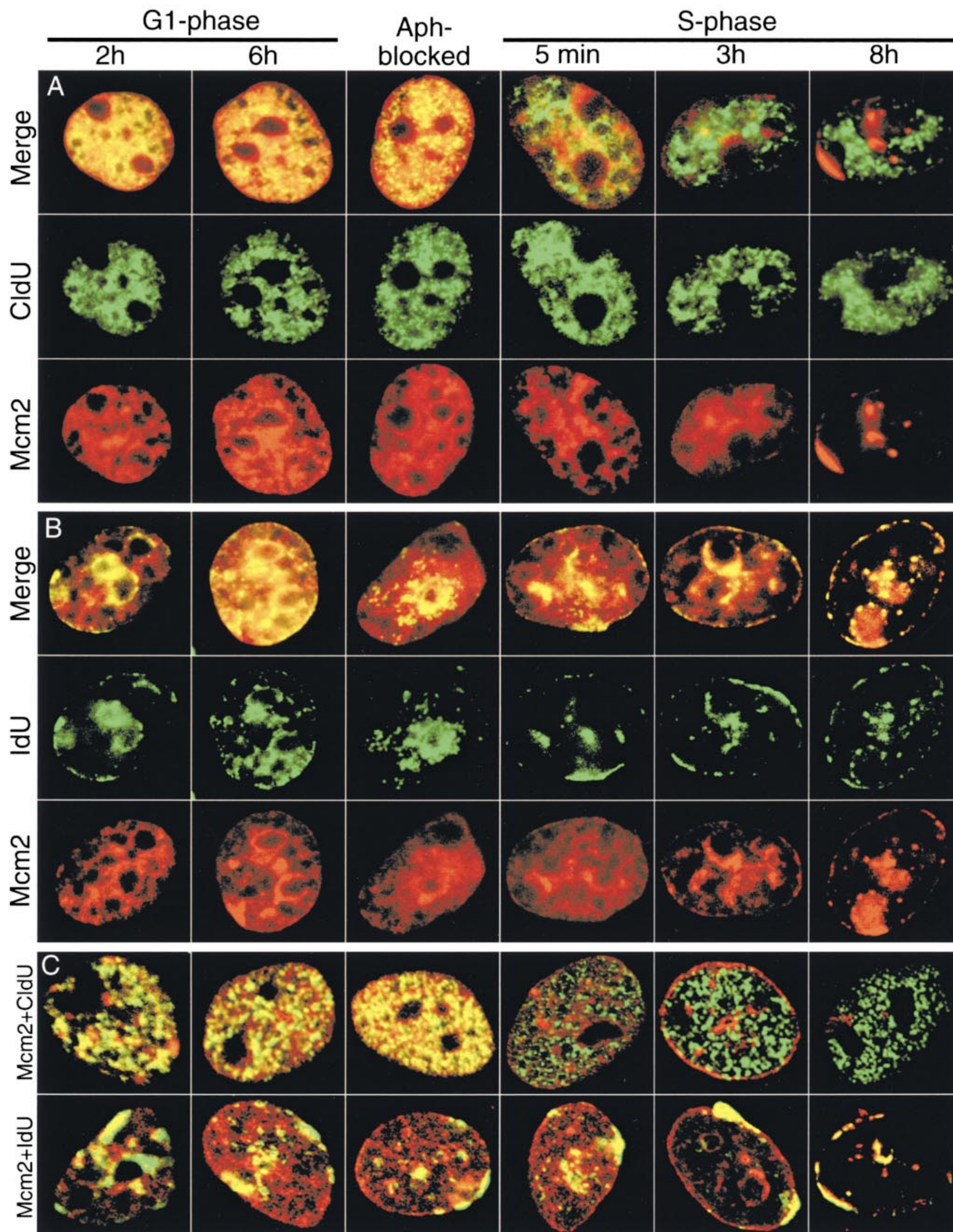


Figure 2.

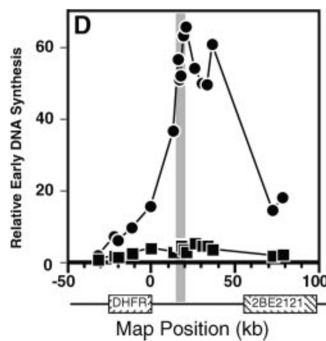


Figure 2. Mcm2 associates with both early-replicating (A and upper row in C) and late-replicating (B and lower row in C) chromatin at the beginning of G1-phase and is released from chromosomal domains at the time when they replicate. CHOC 400 cells, pulse-labeled with CldU during the first 5 min of S-phase and with IdU after 8 h in the same S-phase, were collected in the following mitosis and released in the subsequent cell cycle (see Fig. 1). Aliquots of the cells were then collected at 2 h (pre-ODP) or 6 h (post-ODP) in G1-phase, whereas the remainder of the cells were arrested at the G1/S boundary with aphidicolin and either collected immediately (Aph-blocked) or released for 5 min, 3 h (early-S-phase), or 8 h (late-S-phase). The cells were extracted with 0.5% Triton X-100, fixed with formaldehyde and immunostained with anti-Mcm2 antibody (red) and either anti-CldU (A and upper row in C, green) or anti-IdU (B and lower row in C, green) antibodies as described in Materials and Methods. The images displayed in A and B were obtained by conventional epifluorescence microscopy and were scanned directly from photographs taken with a dual FITC/Rhodamine (Merge) or single FITC (CldU or IdU) or TxRed (Mcm2) fluorescence filters. Shown in part C are single optical sections through cells from the same synchronized cultures obtained by dual-color confocal laser scanning microscopy as described in Materials and Methods. (D) Origin mapping at the DHFR locus by the ELFH assay was performed by introducing aliquots of the same cells, taken at 2 h circles and 6 h squares after release from mitosis, into *Xenopus* egg extract as described in Materials and Methods. The relative amounts of hybridization of the earliest labeled nascent DNA to each probe from the DHFR locus are plotted against the map position of each probe. The horizontal axis includes diagrams of the genomic region encompassed by these probes, including the positions of the DHFR and 2BE2121 genes (Hamlin et al., 1994). The vertical shaded line highlights the positions of probes B-R, which encompass the region of peak initiation activity (Kobayashi et al., 1998; Wang et al., 1998) and references therein).

intensity of anti-Mcm2 staining was generally brighter in later stages of G1-phase. In particular, confocal analysis indicated that the colocalization of Mcm2 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 Mcm2 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 Mcm2 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 Knippers, 1997). Triton extraction of chromatin was slightly more effective at removing soluble Mcm2 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 Mcm2, suggesting that this fraction is tightly associated with chromatin. Consistent with results obtained in HeLa cells (Todorov et al., 1995), Chinese hamster Mcm2 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 Mcm 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 Mcm2 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 Mcm2 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 Mcm2 proteins in Triton-extracted metaphase and anaphase cells did not detect any Mcm2 bound to chromosomes at these stages of mitosis (Fig. 5 E). The appearance of chromatin-bound Mcm2 (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 Mcm2 was undetectable in Triton-treated early-telophase nuclei and weakly detectable in the late-telophase nuclei (Fig. 5 E). We conclude that Mcm2 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 Mcm2 throughout G1-phase, peaking at the start of S-phase. To determine what percentage of the total Mcm2 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 pelleted chromatin (not shown). Results (Fig. 5 B) revealed that, 90 min after metaphase (the pre-ODP stage of G1-phase), only 20% of the total Mcm protein was bound to chromatin. This amount increased to 30% at 6 h after metaphase (post-ODP), 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 Mcm2 with chromatin does not occur as a single defined step at the end of mitosis; instead, there is a continuous loading of Mcm2 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

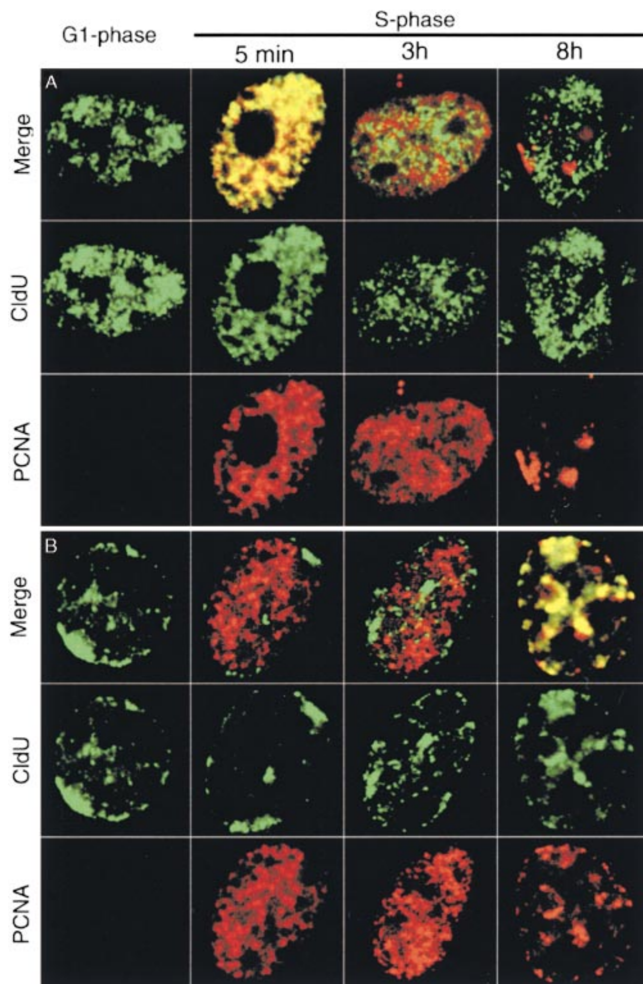


Figure 3. PCNA does not bind nuclear components during G1-phase and associates transiently with chromosomal domains at the time when they replicate. (A) The association of PCNA (red) with the earliest-replicating chromatin regions (CldU-tagged, green) was examined in aliquots of the cells used in the experiment displayed in Fig. 2. (B) Late-replicating genomic regions were pulse-labeled with CldU (green) at 8 h in S-phase in a parallel culture of CHOC 400 cells, mitotic cells were collected few hours later, released in the subsequent cell cycle for the same periods of time and processed in the same way as in Fig. 2.

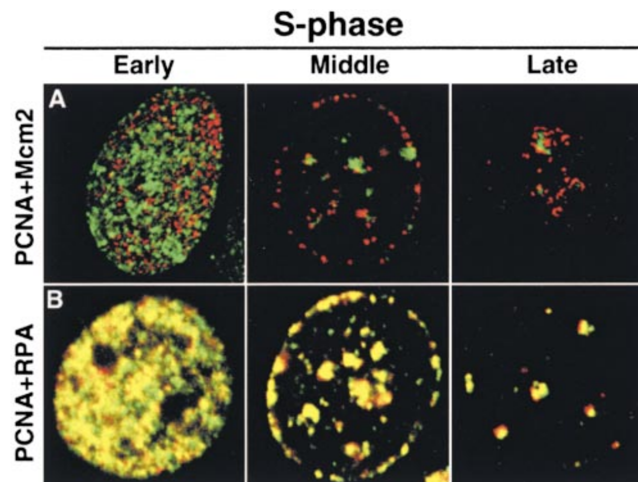


Figure 4. RPA, but not Mcm2, is present at PCNA-containing active mammalian replication forks. Aliquots of the cell cultures (synchronized in early or mid/late S-phase) used in Fig. 2 were immunostained with PCNA-specific (red) and, either Mcm2-specific (A, green), or RPA-specific (B, green) antibodies. Shown are single sections obtained by dual-color confocal laser scanning microscopy.

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 (Krude, 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-

A

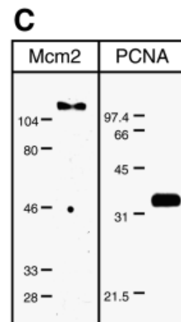
Time after release from nocodazole block	Triton (%) 0 P	0 S	0.5 P	0.5 S	Digitonin (µg/ml) 0 P	70 P	250 P	% of cells in: metaphase	anaphase	telophase and G1-phase
0 min								100	-	-
20 min								95	5	-
40 min								57	24	19
60 min								12	16	72

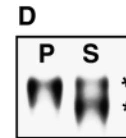
B

Cell cycle phase	Triton (%) 0 P	0 S	0.5 P	0.5 S	Digitonin (µg/ml) 0 P	70 P	250 P	% of Mcm2 in the Triton-resistant nuclear-bound fraction
1.5h in G1-phase (Pre-ODP)								20
6h in G1-phase (Post-ODP)								30
G1/S (aphidicolin block)								45
7h in S-phase								25

F

Cell cycle phase	Triton (%) 0 P	0 S	0.5 P	0.5 S	Digitonin (µg/ml) 0 P	70 P	250 P
1.5h in G1-phase (Pre-ODP)							
6h in G1-phase (Post-ODP)							
G1/S (aphidicolin block)							
3h in S-phase							
7h in S-phase							





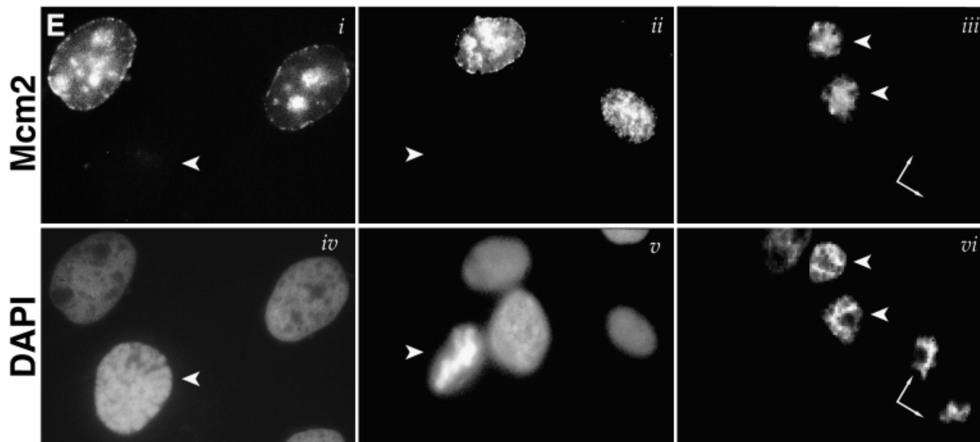


Figure 5. Variations in the amount of chromatin-bound Mcm2 and PCNA during different stages of the cell cycle in CHOC 400 cells. Synchronized cell populations were resuspended in cytoskeleton buffer (for Triton extractions) or transport buffer (for digitonin extractions) and incubated for 5 min on ice with or without addition of permeabilizing agents as described in Materials and Methods. The cellular or nuclear pellets (P) were separated from the soluble fractions (S) by centrifugation. The proteins from each fraction (only the pellets were analyzed in the case of digitonin extractions) were separated by electrophoresis in 10% (A, B, C-Mcm2, and D) or 18% (C, PCNA) SDS–polyacrylamide gels (amounts corresponding to 2×10^5 cells were loaded in each lane), transferred to nylon membranes and probed with an anti-Mcm2 antibody (A–D) or anti-PCNA antibody (C, PCNA and F). (A) Immunoblotting analysis of chromatin-bound Mcm2 at 20, 40, and 60 min after release of CHOC 400 cells from metaphase block. The percentages of cells in different stages of mitosis, determined microscopically after staining aliquots of the synchronized cells with 0.1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI), are indicated on the right for each time point. (B) Immunoblot analysis of chromatin-bound Mcm2 at different times during interphase. The permeabilization of cells with 70 µg/ml digitonin produces nuclei with intact membranes and can be used as a reference for the total amount of 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 CHOC400 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 slow moving form is detected in the nuclear pellet fraction. (E) Immunofluorescent analysis of Mcm2 chromatin-binding in Triton-extracted CHOC 400 cells at different stages of mitosis. Asynchronous 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.

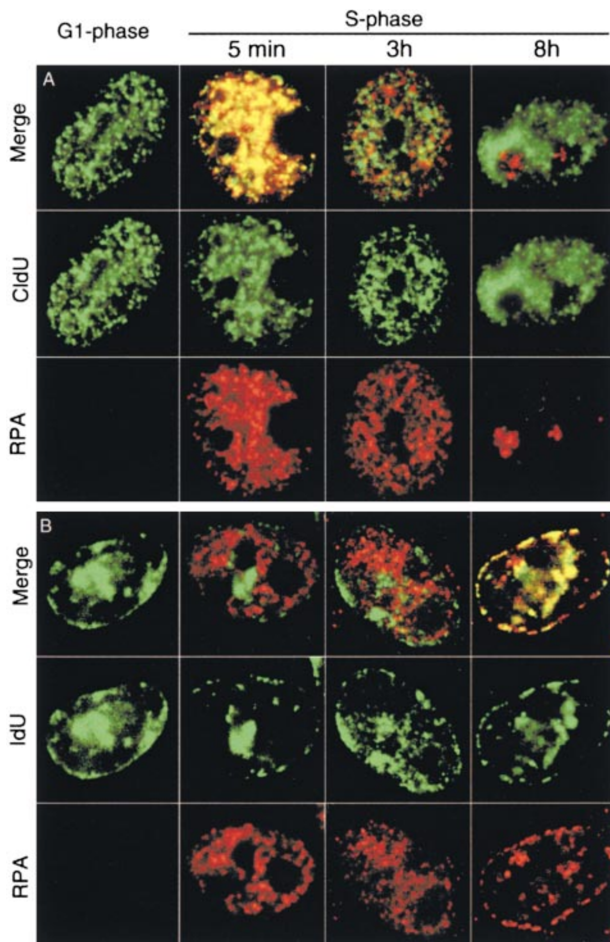


Figure 6. Hamster RPA does not assemble into detergent-resistant pre-RC foci during early G1-phase and associates transiently with chromosomal domains at the specific time of S-phase when they replicate. The association of RPA (red), with earliest-replicating (CldU, green, A) or late-replicating (IdU, green, B) chromatin regions was examined in aliquots of the cells used in Fig. 2. Immunolabeling was performed as in Fig. 2.

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 CHOC 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. As 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. At 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, CHOC 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

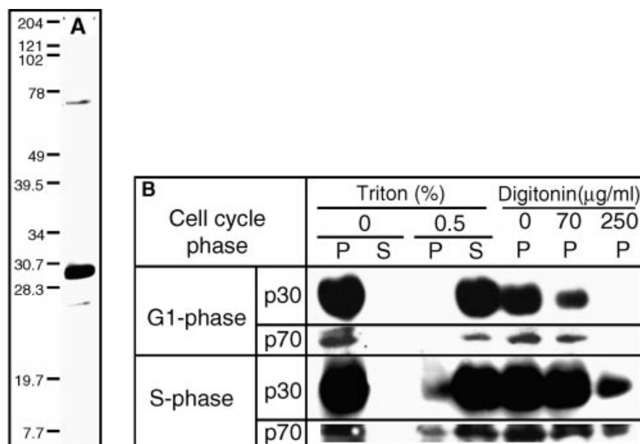


Figure 7. Immunoblot analysis of chromatin-bound RPA during G1 and S-phase. (A) Proteins in a total extract from CHOC 400 cells (2.5×10^5 cells) were separated by electrophoresis in 12.5% SDS–polyacrylamide gels, transferred to a nylon membrane and probed with anti-RPA antibodies. The rabbit polyclonal anti-RPA serum [raised against human RPA trimeric complex (Din et al., 1990)] recognized protein bands corresponding to the large (apparent molecular mass of ~70 kD), middle (~30 kD), and small (~14 kD, a very faint band invisible on this blot) RPA subunits. Positions of molecular mass standards (indicated in kD) are marked on the left. (B) Western blots of chromatin-bound RPA in early G1 and S-phase. Aliquots of the same protein extracts from synchronized CHOC 400 cells used in Fig. 5, B and F, were subjected to SDS-PAGE (12.5% polyacrylamide gels, equivalents of 2×10^5 cell per lane), transferred to nylon membranes and probed with anti-RPA polyclonal antibodies. Only results for the middle (p30) and large (p70) RPA subunits are displayed, since the signal for the small RPA subunit was very weak (we have found no evidence for disassembly of the RPA complex during the cell cycle in control immunolocalization experiments, using monoclonal antibodies specific for the individual RPA subunits together with the polyclonal serum against the heterotrimeric RPA complex; Dimitrova, D.S., and D.M. Gilbert, manuscript in preparation). An increase of total cellular RPA protein and a maximal level of RPA in the nuclear pellet fraction was detected during early S-phase (see Fig. 5 F).

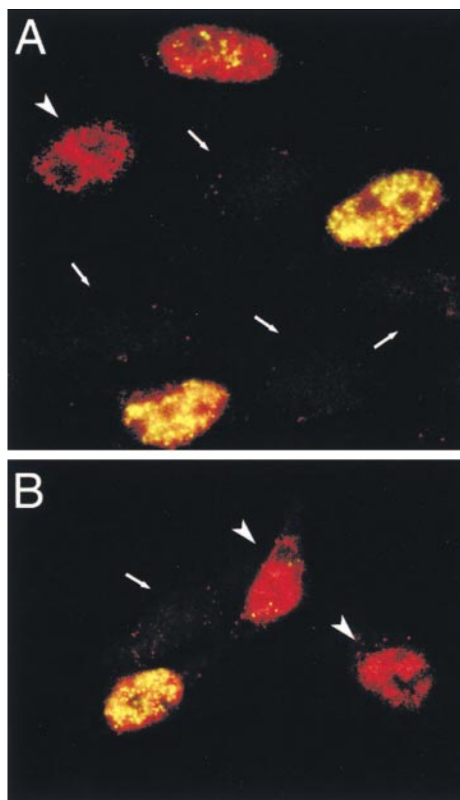


Figure 8. Hamster RPA and PCNA are assembled into detergent-resistant replicative complexes within less than an hour from the start of S-phase. CHOC 400 cells, synchronized in mitosis, were released for 8 h in the following cell cycle (20% of the cells had entered S-phase at this time). The cells were pulse-labeled with 100 μM CldU for 1 min, extracted with Triton and fixed with formaldehyde as described in Materials and Methods. Aliquots of the cells were stained with anti-CldU antibodies (green) and either anti-RPA antibodies (red, A) or anti-PCNA antibodies (red, B). S-phase cells display yellow coloration resulting from the colocalization of RPA or PCNA (red) with nascent DNA (CldU, green). Arrowheads point to nuclei that exhibit positive staining for RPA or PCNA, but no CldU label. Arrows indicate the positions of G1-phase cells (barely visible as faint shadows).

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 (Newlon, 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 (Krude, 1995; Todorov et al., 1995; Romanowski 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 replicon 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 replicons 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 replicons shortly 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. 4 A). 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 a key participant in the initiation step of DNA replication. Several groups (Adachi and Laemmli, 1992, 1994; Kubota et al., 1995; Yan and Newport, 1995a; Coleman et al., 1996; Coue et al., 1996) have shown that, 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 CHOC 400 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 replicon 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 assemble at the G1/S transition in human fibroblasts (Kill et al., 1991; Hozak et al., 1994) and contain both PCNA and DNA polymerase α. Our results are also consistent with recent chromatin immunoprecipitation studies in *S. cerevisiae* that did not detect any association of RPA with yeast chromatin until the onset of S-phase (Tanaka and Nasmyth, 1998). We suggest that previous data on G1- or G2-phase nuclear association of RPA proteins (Brenot-Bosc et al., 1995; Murti et al., 1996) derive from the presence of a soluble, detergent-extractable form of RPA (Dimitrova, D.S., 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 (Tsurimoto 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 (Adachi and Laemmli, 1994; Yan and Newport, 1995b). The assembly of RPA foci onto 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 (Adachi 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 *Xenopus* 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, 1995b). 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 Loeb, 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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