Dynamics of pre-replication complex proteins during the cell division cycle

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Replication of the human genome every time a cell divides is a highly coordinated process that ensures accurate and efficient inheritance of the genetic information. The molecular mechanism that guarantees that many origins of replication fire only once per cell-cycle has been the area of intense research. The origin recognition complex (ORC) marks the position of replication origins in the genome and serves as the landing pad for the assembly of a multiprotein, pre-replicative complex (pre-RC) at the origins, consisting of ORC, cell division cycle 6 (Cdc6), Cdc10-dependent transcript (Cdt1) and mini-chromosome maintenance (MCM) proteins. The MCM proteins serve as key participants in the mechanism that limits eukaryotic DNA replication to once-per-cell-cycle and its binding to the chromatin marks the final step of pre-RC formation, a process referred to as 'replication licensing'. We present data demonstrating how the MCM proteins associate with the chromatin during the G1 phase, probably defining pre-RCs and then anticipate replication fork movement in a precisely coordinated manner during the S phase of the cell cycle. The process of DNA replication must also be carefully coordinated with other cell-cycle processes including mitosis and cytokinesis. Some of the proteins that control initiation of DNA replication are likely to interact with the pathways that control these important cell-cycle transitions. Herein, we discuss the participation of human ORC proteins in other vital functions, in addition to their bona fide roles in replication.

Keywords: DNA replication; mini-chromosome maintenance proteins; origin recognition complex; pre-replication complex; proliferating cell nuclear antigen; S phase

1. INTRODUCTION

DNA replication in eukaryotic cells is a complex process that requires the precise coordination of numerous proteins and leads to the duplication of the genetic information for the next cell generation. This highly regulated process requires the ordered assembly of several proteins at the origins of DNA replication to form a competent, pre-replicative chromosomal state (Stillman 1996; Kelly & Brown 2000; Bell 2002; Bell & Dutta 2002; Blow & Hodgson 2002). The molecular mechanisms ensuring the accurate initiation of DNA replication, particularly it being restricted to the S phase and occurring only-once-per cell-cycle, have been major areas of research. The mechanisms that ensure that no stretch of the genome is left unreplicated beyond the normal duration of the S phase are also not clearly understood, but are in part linked to cell-cycle checkpoint controls (Zhou & Elledge 2000). In addition, there must exist stringent regulatory mechanisms that couple DNA replication to other events in the process of cell division, such as mitosis and cytokinesis.

The very large size of eukaryotic genomes demands that they are duplicated from multiple origins of DNA replication distributed along the entire length of each chromosomal DNA molecule. Activation of each replication origin leads to the assembly of a bi-directional replication fork (Diffley 1996; Waga & Stillman 1998; Zhou & Elledge 2000). The formation of a pre-RC at the origins occurs by the ordered assembly of several replication proteins, although in human cells it is not known whether this process at each origin is directed by specific DNA sequences (DePamphilis 1999). In the yeast *Saccharomyces cerevisiae*, the ORC, a six subunit initiator protein (Bell & Stillman 1992) provides a molecular landing pad for the sequential assembly of Cdc6, Cdt1 and MCM proteins (Bell 2002). This pre-RC is assembled during exit from mitosis, and origin firing is triggered in a sequential manner by the action of at least two sets of protein kinase activities: the cyclin-dependent protein kinases and Dbf4p-Cdc7p (Henneke *et al.* 2003). After initiation, the protein complex at each origin changes to a post-replication state (post-RC), thereby preventing further initiation events from the same origins for the rest of the cell cycle (Kelly & Brown 2000; Bell & Dutta 2002). In vertebrate cells, but not in yeast, another protein called geminin also contributes to avoid re-replication by preventing the loading of MCM proteins onto chromatin (McGarry & Kirschner 1998; Wohlschlegel *et al.* 2000; Dhar *et al.* 2001b; Tada *et al.* 2001; Hodgson *et al.* 2002; Yanagi *et al.* 2002).

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Figure 1. Dual colour immunostaining of PCNA and MCM serves as a good G1 marker. (a) IF was used to analyse chromatin-bound PCNA ((i) green) and MCM2 ((ii) red) after a pre-extraction procedure in HeLa cells (Martini *et al.* 1998). Merge is shown in (iii). An arrowhead denotes a PCNA negative, MCM positive G1 cell. Scale bar, 10 μ m. Before fixation and IF, cells were extracted with 0.5% Triton X-100, fixed in paraformaldehyde and then in methanol at -20°C (Martini *et al.* 1998). (b) MCM binding to chromatin precedes PCNA during S phase. Dual IF of chromatin-bound PCNA ((i), (iv) and (vii) green) and MCM3 ((ii), (v) and (viii) red), during S phase, shown during early S phase ((i)–(iii)), PCNA had a punctate pattern (i), whereas MCM3 had already decorated the pericentric heterochromatin (ii). During mid S phase based on PCNA staining (iv), MCM3 staining pattern was reminiscent of the late S phase and by late S based on PCNA (vii), MCM3 was almost off the chromatin (viii). Chromatin was stained with DAPI ((iii), (vi) and (ix) blue). Scale bar, 5 μ m.

Yeast cells were employed for cell-cycle studies because their shape revealed the stage of the cell division cycle. In a population of asynchronously growing mammalian cells, it has not been easy to identify the specific cell-cycle stage of each individual cell. There are excellent molecular markers for S-phase progression, the best being incorporation of BrdU or staining of cells with antibodies against the DNA replication fork protein PCNA (O'Keefe *et al.* 1992). It has been difficult, however, to distinguish individual cells that are in the G1 or G2 stages of the cell division cycle because no nuclear DNA synthesis is occurring in these cells. We have used differential immunostaining patterns of pre-RC proteins in mammalian cells to determine the stage of the cell division cycle in fixed cells.

In so doing, we have uncovered new functions for some of the ORC subunit proteins.

2. DYNAMICS OF S PHASE: VARIED PATTERNS OF MINI-CHROMOSOME MAINTENANCE AND PROLIFERATING CELL NUCLEAR ANTIGEN IN S PHASE

MCM proteins are conserved throughout evolution, from Archaea to Eukarya, and are essential for the initiation of DNA replication (Tye 1999; Laskey & Madine 2003). In eukaryotes, Mcm2–7 are a group of six related proteins that form a hexamer structure that is likely to function as a replicative helicase. They have been

Figure 2. Chromatin association of PCNA and MCM proteins in MCF-7 cells. Dual colour IF approach to study chromatin-bound PCNA (*a(i)–h(i)* green) and MCM3 (*a(ii)–h(ii)* red) revealed strong MCM3 staining in PCNA negative G1 cells (*a(i)–(iii)*). With the entry into S phase both PCNA and MCM3 showed a punctate nuclear staining (*b(i)–(iii)*). During early S phase, while PCNA showed a rather homogenous nuclear staining, MCM3 seemed to decorate the pericentric heterochromatin (*c(i)–(iii)*, *d(i)–(iii)*). During mid S phase, while PCNA showed the typical heterochromatic association, MCM3 staining decreased and seemed to be restricted to a few spots (*e(i)–(iii)*, *f(i)–(iii)*). By late S, when PCNA is present in a few spots, most of MCM3 is lost from the chromatin (*g(i)–(iii)*), and by G2 both PCNA and MCM3 were absent from the chromatin (*h(i)–(iii)*). MCM3 and PCNA staining in conjunction provide excellent markers to distinguish different stages of the cell cycle. Chromatin was stained with DAPI (*a(iii)–h(iii)* blue), in blue. Scale bar, 5 μ m.

shown to be key participants in the mechanism that limits replication to once-per-cell-cycle (Tye 1999). The association of the MCM proteins with chromatin (Rowles & Blow 1997; Coverley *et al.* 2000; Madine *et al.* 2000) occurs during late telophase or early G1 in yeast (Diffley *et al.* 1994; Donovan *et al.* 1997; Liang & Stillman 1997; Tanaka *et al.* 1997) as well as in mammalian cells (Dimitrova *et al.* 1999, 2002; Mendez & Stillman 2000). At this time in the cell cycle, there is no cyclin-dependent kinase activity, which inhibits pre-RC formation (Piatti *et*

al. 1996; Noton & Diffley 2000). After the G1/S transition, MCMs dissociate progressively from the chromatin during the S phase and G2 (Todorov *et al.* 1995; Fujita *et al.* 1996; Krude *et al.* 1996; Holthoff *et al.* 1998; Ritzi *et al.* 1998; Mendez & Stillman 2000).

To gain insight into the distribution pattern of MCM proteins during different cell-cycle stages, we performed immuno-localization of MCM proteins after cells had been treated with detergent before fixation. The detergent pre-extraction step removed most of the soluble nuclear

Figure 3. PCNA and MCMs do not co-localize on chromatin. High-resolution dual immunolocalization of chromatin-bound PCNA (*a(i)–c(i)*) and MCM3 (*a(ii)–c(ii)*) red suggests that PCNA surrounds the MCM3 (*a(iii)–c(iii)*). The images were obtained by using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) on an Olympus microscope with a $63 \times 1.4\text{NA}$ objective. Z15, 20 and 32 represent the three optical sections of the same cell.

proteins, but retained the chromatin-bound proteins (Mendez & Stillman 2000). The DNA replication fork protein PCNA was used to identify cells in the S phase. In the two different human cell-lines analysed, HeLa (figure 1) and MCF-7 (figure 2), dual IF analysis of the chromatin-bound MCM and PCNA served as an excellent marker to discriminate G1 cells within a cell population. Cells that showed an intense and punctate MCM staining pattern and were not stained with anti-PCNA antibodies after detergent extraction (figures 1 and 2*a*) can be confidently considered G1 cells. Most of the MCM proteins were extracted from the nuclei under these conditions, leaving a sub-fraction associated with a component of the nucleus that was made soluble upon treatment with micrococcal nuclease (data not shown). This fraction of the MCM protein complex may serve as the replicative helicase, although this hypothesis is hard to reconcile with the observation (reproduced below) that MCM proteins do not colocalize with DNA replication foci in vertebrate cells (Madine *et al.* 1995; Krude *et al.* 1996; Dimitrova *et al.* 1999). However, yeast MCM proteins are necessary for passage through the S phase and presumably function at DNA replication forks (Labib *et al.* 2000). A possible resolution of this paradox that has recently been proposed suggests that MCMs act as rotary 'DNA pumps' at a distance from replication forks (Laskey & Madine 2003).

As previously reported, specific PCNA staining patterns are observed at different times during the S phase (Bravo & Macdonald-Bravo 1987; O'Keefe *et al.* 1992). Thus the PCNA staining pattern was used as a marker when analysing the pattern of IF staining of chromatin-

associated MCM proteins at different stages of the S phase. The MCM proteins were not concentrated at the replication forks marked by PCNA, as described in earlier studies (Madine *et al.* 1995; Krude *et al.* 1996; Dimitrova *et al.* 1999). However, an interesting pattern emerged from our studies based on the classical S-phase patterning using PCNA or BrdU (O'Keefe *et al.* 1992). In cells that had just entered the S phase (see the early PCNA pattern, figure 2*b(i)*), chromatin-bound MCM3 was uniformly distributed in the nucleus just as it was in the G1 phase of the cell cycle (figure 2*b(ii)*). After the transition into the next early S-phase PCNA pattern (see PCNA pattern figures 1*b(i)* and 2*c(i)*), the MCM3 concentrated around the nucleoli and on the rim of the nuclear membrane (figure 2*c(ii)*) possibly suggesting a predominant interaction with heterochromatin (figures 1*b(ii)–(iv)* and 2*c(ii), d(ii)*). This pattern was similar to that observed for PCNA staining or BrdU incorporation in mid S-phase cells (compare figure 2*c(ii)* with 2*f(i)*). Once the cells proceeded into mid S phase (figures 1*b(iv)* and 2*e(i), f(i)*), the MCM3 staining gradually decreased and was concentrated in a few spots, a pattern that resembles that of PCNA in late S-phase cells (compare figure 1*b(v)* with (vii) and figure 2*e(ii)* or *f(ii)* with *g(i)*). Subsequently, when the cells progressed into late S phase based on the PCNA staining pattern, MCM staining was almost completely lost (figures 1*b(vii)–(ix)* and 2*g(i)–(iii)*). The G2 nuclei could be identified by the absence of PCNA and MCM staining (figure 2*h(i)–(iii)*). High resolution, deconvolution images of MCM and PCNA dual localization corroborated the above observations (figure 3). In the early S-phase nuclei it was

Figure 4. Appearance of MCMs in the post-mitotic daughter nuclei. (a) IF revealed that MCM3 enters the daughter nuclei during the late telophase ((iii), (iv)). Note the newly forming daughter nuclei devoid of MCM3 during early telophase ((i), (ii)). (b) Nuclear envelope assembly (Nu.pore protein p62, (ii), (v)) was concomitant with the appearance of MCM4 in the post-mitotic daughter nuclei ((i), (iv)). Note that MCM4 was not present in the daughter nuclei during early telophase (i) before nuclear envelope assembly (ii). Chromosomes were stained with DAPI ((iii), (vi)). Scale bar, 5 μ m. In this figure, cells were not pre-extracted with detergent before IF.

apparent that PCNA (green) seemed to surround the MCM foci, but no apparent overlap was observed (figure 3). The abundance and distribution of the MCM proteins suggest that they may not function directly at the replication forks and may have additional roles besides the putative helicase activity.

The results suggest that the temporal pattern of nuclear localization of MCM proteins during the S phase precedes that of PCNA and that the MCM proteins could somehow serve as a guide for the movement of the replication forks. Alternatively, it could be possible that the euchromatic pre-RCs were assembled during early G1, whereas the heterochromatic pre-RCs were assembled during early S phase. It has been suggested that differential loading of MCM proteins onto early—versus late—replication origins might be responsible for the temporal order of

chromosomal DNA replication (Kearsey & Labib 1998). However, Dimitrova *et al.* (1999) showed that MCM loading onto chromatin onto both early- and later-replicating chromatin occurs from late telophase throughout G1. If the temporal pattern of chromatin-bound MCM observed here reflects loading of MCM proteins onto late-replicating regions during the S phase, then such loading would have to be unaffected by the active cyclin-dependent protein kinases that are known to block MCM loading (Diffley 1996; Stillman 1996; Bell & Dutta 2002). Nevertheless, the current observations support the idea that after initiation of DNA replication, the MCM proteins preferentially bind to unreplicated DNA and not to the replicating or replicated DNA. The displacement of MCM proteins from replicated DNA probably contributes to processes that prevent re-replication.

Figure 5. End-on view of human Orc6 in a metaphase cell. Orc6 localization revealed kinetochore association (punctate spots), reticulate membraneous staining and presence at the midzone, as viewed in the rosetta confirmation of a metaphase cell. DNA was visualized with DAPI (red).

In a recent study, the MCM and PCNA proteins were found to colocalize to the sites of amplification of the *Drosophila* chorion gene amplicons (Claycomb *et al.* 2002). This analysis suggests that the MCM proteins might be associated with the DNA replication fork, but this system is unusual because multiple initiations of DNA replication occur at a single locus. Thus the mechanism of re-initiation from a fixed origin might demand that MCM proteins be constitutively bound to the start sites of fork progression.

The dual staining of human cells with MCM and PCNA antibodies provided a convenient method to determine at the single cell level the precise time during the cell cycle when the cell was fixed. G1 phase cells were MCM⁺ and PCNA⁻, S-phase cells showed a varied and temporally regulated pattern of double-positive staining for both MCM and PCNA, with the MCM staining pattern preceding the PCNA pattern. G2 cells were MCM⁻ and PCNA⁻. Thus staining asynchronous cells with both MCM and PCNA antibodies readily distinguishes the stage at which an individual cell exists in the cell division cycle.

3. COUPLING DNA REPLICATION AND CELL-CYCLE PROGRESSION

Chromatin is virtually devoid of MCM proteins during G2 and throughout most of mitosis. The absence of MCM3 and MCM4 proteins in daughter nuclei or chromatin present in early telophase is shown in figure 4a(i), (ii) and b(i). During late telophase, the MCM proteins abruptly relocated within the newly formed daughter nuclei (figure 4a(iii)–(iv), b(iv)–(vi)). Staining of cells with anti nuclear pore antibodies showed that the appearance of MCM4 in the daughter nuclei during late telophase occurred about the time of nuclear envelope formation (figure 4b). Pre-extraction procedures to remove the

soluble MCM fraction from cells at this stage of the cell cycle showed that the MCM proteins were associated with the chromatin during early G1 (data not shown). Binding of the MCM proteins onto chromatin represents the time when functional licensing of chromosomes occurs in rapidly proliferating human cells in culture.

As stated above, it has been proposed that the MCM proteins act as a DNA helicase at the DNA replication forks, but this idea is incompatible with the different localization of PCNA and MCM proteins during S phase. To resolve this paradox, Laskey & Madine (2003) have proposed a 'rotary pump' model in which the hexameric MCM complexes unwind the DNA at a site distant from the site of DNA synthesis. We propose an alternative model in which the MCM proteins are involved in remodelling of the nucleosome structure before replication of the locus by acting as a fixed DNA translocase much like the RuvB double hexamer at recombination junctions (Sharples *et al.* 1999; Caruthers & McKay 2002). As DNA from both sides of the replication origin is pulled through the MCM double hexamer, the histone H2A.H2B dimers that were present on the nucleosomes would be removed, causing local unwinding of the DNA. Because there exists one negative supercoil stored in every nucleosome, for every histone H2A.H2B dimer so removed, topological equivalence would allow 10 bp of DNA to be unwound. Thus remodelling of a segment containing only 10–20 nucleosomes would unwind 100–200 bp of DNA, sufficient for an Okazaki fragment size replication unit. If more nucleosomes were remodelled by removing H2A.H2B dimers, then a large region of DNA would be unwound and perhaps stabilized by replication protein A (RPA), the single-strand DNA binding protein. We further imagine that as the duplex DNA is replicated, the tetramer of histones H3.H4 that are still associated with the DNA would be temporarily displaced, stored on the MCM protein complex and then reloaded onto one of the replicated sister DNA molecules before reassembly of nucleosomes by a chromatin-assembly factor-dependent assembly mechanism (Smith & Stillman 1989, 1991; Verreault 2003). Such a mechanism is consistent with the observations that MCM proteins bind histones H3 and H4 (Ishimi *et al.* 1996; Holland *et al.* 2002). This model, and that proposed by Laskey & Madine (2003) are also consistent with the structural descriptions of an Archaea MCM dodecamer, a ring-shaped molecule with a central channel that is large enough to accommodate double-stranded DNA (Chong *et al.* 2000; Fletcher *et al.* 2003). Coupled remodelling of the histones and translocation of the duplex DNA through the MCM hexamer to an unwound state at sites distal to the DNA replication fork machinery could explain the dynamic patterns of MCM localization in the nuclei of S-phase cells that are shortly followed by a similar pattern of localization of the replication fork protein PCNA.

4. ORIGIN RECOGNITION COMPLEX 6: A MULTI-FUNCTIONAL PROTEIN

ORC plays a pivotal function during the chromosome replication cycle by binding to DNA and initiating the process of replication licensing and subsequent DNA replication. Interestingly, ORC in the budding yeast *S. cerevisiae*

Figure 6. Orc6 assembles at the kinetochore before the nuclear envelope breakdown. The punctate pattern of Orc6 at this stage represents kinetochore localization (Prasanth *et al.* 2002). Orc6 (red) appeared at the kinetochores (*a(i)* and *c(i)*) during late G2/early prophase, before the breakdown of nuclear envelope, as visualized by lamin (*a(ii)* green) and nuclear pore protein p62 (*c(ii)* green). The kinetochore association of Orc6 (*b(i)* and *d(i)*) is more apparent following nuclear envelope disassembly (*b(ii)* and *d(ii)*). Kinetochore association of Orc6 was concomitant with appearance of Bub1 on the kinetochore, which is reported to be during late G2 (data not shown). DNA was visualized with DAPI (*a(iii)*–*d(iii)* blue). Scale bar, 10 μ m.

Figure 7. Nuclear envelope is assembled after the entry of Orc6 protein into the daughter nuclei. Orc6 (*a(i)* red) appeared in the post-mitotic daughter nuclei before the establishment of nuclear envelope, as visualized by staining the nuclear pore protein p62 (*a(ii)* green). The level of Orc6 protein (*b(i)*) increased in the daughter nuclei with the formation of nuclear envelope (*b(ii)*). The arrowhead in (*a(i)*) denotes the midbody association of Orc6 during telophase. It is not very clearly visible in (*b(i)*) owing to the different focal length used to capture the nuclear pore protein. Scale bar, 5 μ m.

has also been implicated in transcriptional gene silencing and in *Drosophila*, Orc2 is known to be associated with heterochromatin (Pak *et al.* 1997; Bell 2002; Gerbi &

Bielinsky 2002). Orc2 mutants in *Drosophila* (Loupart *et al.* 2000; Pflumm & Botchan 2001) and depletion of Orc2 in human cells (S. Prasanth and B. Stillman, unpublished

data) show cells arrested with abnormally condensed chromosomes and with spindle defects. Furthermore, MCM mutants and MCM depletion in *Drosophila* (Pflumm & Botchan 2001; Christensen & Tye 2003), as well as Mcm10 and Cdc45 depletion (Christensen & Tye 2003) also resulted in aberrant chromosome condensation, supporting the suggestion that DNA replication and chromosome condensation are intimately linked processes (Pflumm 2002). These results suggested that ORC and other DNA replication proteins might coordinate multiple stages of the chromosome replication and segregation cycle. By studying the function of the individual ORC subunits, we have uncovered unexpected roles for ORC proteins during the process of cell division itself.

Recent studies indicate that ORC may be a more dynamic molecule than previously thought (Loupert *et al.* 2000; Chesnokov *et al.* 2001; Pflumm & Botchan 2001; Prasanth *et al.* 2002). Studies of mammalian cells suggest that not all the ORC subunits are tightly associated in a single, heterohexameric complex throughout the cell division cycle. The largest subunit of ORC, Orc1 was removed from the chromatin as cells progressed into S phase and rebound to chromatin only as cells progressed into late telophase (Natale *et al.* 2000; Kreitz *et al.* 2001; Li & DePamphilis 2002; Mendez *et al.* 2002). Biochemical studies addressing the assembly of human ORC found that a core subcomplex consisting of Orc2–5 was formed in insect cell extracts that had been infected with recombinant baculoviruses expressing individual ORC subunits (Dhar *et al.* 2001a; Vashee *et al.* 2001). An apparent six subunit complex, including Orc1 and possibly the Orc6 subunit could be immunoprecipitated under certain conditions but the complex was unstable compared with the yeast ORC (Dhar *et al.* 2001a; Vashee *et al.* 2001).

Orc6 protein has been found to be highly dynamic with respect to its cell cycle localization in human cells (Prasanth *et al.* 2002). The human Orc6 is similar in primary sequence to the *Drosophila* Orc6 (DmOrc6) and most of the DmOrc6 that is present in the embryo exists separately from the six subunit ORC (Chesnokov *et al.* 2001). The human Orc6 protein localizes to nuclear chromatin during interphase, but it was found to have a novel localization pattern during mitosis. As cells progressed from prophase through anaphase, some Orc6 decorated the outer kinetochore at centromeres, and the remaining Orc6 protein localized in a reticular pattern along the plane of cell division (Prasanth *et al.* 2002; figure 5). After the metaphase to anaphase transition, some of the Orc6 ended up in the midbody of the cell, a structure that defines the last connection between the sister cells (figure 7).

The association of the Orc6 protein at the kinetochore occurred during the G2/prophase transition and occurred before the breakdown of nuclear lamina (figure 6a(i)–(iii), b(i)–(iii)) and nuclear envelope (figure 6c(i)–(iii), d(i)–(iii)). Orc6 protein redistributed into the daughter nuclei at the end of mitosis and this occurred well before the nuclear envelope was laid down (figure 7a(i)–(iii), b(i)–(iii)). In addition to its novel localization, Orc6 was shown to function in regulating chromosomal segregation and cytokinesis, as inhibition of Orc6 expression by siRNA resulted in cells accumulating with multinucleated phenotype (Prasanth *et al.* 2002). Cells that lacked Orc6 had

decreased levels of DNA replication, which became apparent after the cytokinesis defect, suggesting that small pools of Orc6 protein were sufficient to maintain its replication function.

The observation that Orc6 associated with both kinetochores at centromeres and with the plane of cell division during mitosis suggests that it may function to coordinate DNA replication with the processes of chromosome segregation and cytokinesis. Coordination between DNA replication and cell division is also carefully regulated in bacteria (see Sherratt *et al.* 2004).

5. CONCLUDING REMARKS

Despite the significant advances in our understanding of the initiation and control of DNA replication, numerous questions remain unanswered. It is crucial to understand how the components of the pre-RC regulate chromatin structure at or near sites of DNA synthesis and whether they also influence higher-order chromosome structure features such as condensation and cohesion. It would also be interesting to know why there is a vast excess of the MCM proteins relative to the amounts of other replication fork proteins. The assembly of proteins at the pre-RC so far is not well understood and the molecular events leading to unwinding of the origin DNA and the recruitment of the DNA synthesis machinery remain largely speculative. The surprising dynamics of some of the ORC subunits in mammalian cells suggest that these proteins may function to coordinate many of the processes that combine to duplicate and segregate chromosomes to daughter cells, but much more needs to be done to understand how this might occur. So even 50 years after the elucidation of the double helix structure of DNA, how the genetic material is inherited remains a major topic of investigation.

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GLOSSARY

- BrdU: bromo-deoxyuridine
 Cdc6: cell division cycle 6
 Cdt1: Cdc10-dependent transcript
 DAPI: 4',6-diaminidino-2-phenylindole
 Dbf4p-Cdc7p: dumb bell former 4-cell division cycle 7
 IF: immunofluorescence
 MCM: mini-chromosome maintenance
 ORC: origin recognition complex
 PCNA: proliferating cell nuclear antigen
 pre-RC: pre-replicative complex