Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases

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Eukaryotic cells use multiple replication origins to replicate their large genomes. Some origins fire early during S phase whereas others fire late. In *Saccharomyces cerevisiae*, initiator sequences (ARSs) are bound by the origin recognition complex (ORC). Cdc6p synthesized at the end of mitosis joins ORC and facilitates recruitment of Mcm proteins, which renders origins competent to fire. However, origins fire only upon the subsequent activation of S phase cyclin-dependent kinases (S-CDKs) and Dbf4/Cdc7 at the G₁/S boundary. We have used a chromatin immunoprecipitation assay to measure the association with ARS sequences of DNA primase and the single-stranded DNA binding replication protein A (RPA) when fork movement is inhibited by hydroxyurea (HU). RPA's association with origins requires S-CDKs, Dbf4/Cdc7 kinase and an Mcm protein. The recruitment of DNA primase depends on RPA. Furthermore, early- and late-firing origins differ not in the timing of their recruitment of an Mcm protein, but in the timing of RPA's recruitment. RPA is recruited to early but not to late origins in HU. We also show that Rad53 kinase is required to prevent RPA association with a late origin in HU. Our data suggest that the origin unwinding accompanied by RPA association is a key step, regulated by S-CDKs, Dbf4/Cdc7 and Rad53p. Thus, in the presence of active S-CDKs and Dbf4/Cdc7, Mcms may open origins and thereby facilitate the loading of RPA.

Keywords: CDK/DNA primase/Mcm/Rad53/RPA

Introduction

The replication of the large genomes of eukaryotic cells is made possible by the use of multiple replication origins per chromosome. Multiple origins must, however, be very carefully regulated if all chromosomal sequences are to be replicated once and only once during each cell division cycle. Some origins fire early during S phase whereas others fire late, but none fires more than once, except in rare cases where cells undergo endoduplication (reviewed in Diffley, 1996; Nasmyth, 1996; Stillman, 1996). Bi-directional replication is only initiated at specific chromosomal sites, called autonomously replicating sequences (ARSs) in *Saccharomyces cerevisiae* (reviewed in Campbell and Newlon, 1991), to which first the origin recognition complex (ORC), then Cdc6p, and finally the Mcm complex (consisting of Mcm2–7 proteins) have been

recruited (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). ORC, Cdc6p and Mcm proteins also associate with chromatin in this order, in both yeast and *Xenopus* (Coleman *et al.*, 1996; Romanowski *et al.*, 1996; Rowles *et al.*, 1996; Donovan *et al.*, 1997; Liang and Stillman, 1997). Initiation subsequently requires activation of two kinds of kinases, which occurs around the same time as the firing of early origins (reviewed in Diffley, 1996; Nasmyth, 1996; Stillman, 1996). These kinases include S-phase cyclin-dependent kinases (S-CDKs) whose activity depends on cyclin regulatory subunits (Cln5 and 6 in *S.cerevisiae*, cyclin E and A in animal cells) and Cdc7p whose activity depends on a regulatory subunit Dbf4p. It is not well understood how the firing of late origins is delayed. Each new round of replication requires a cycle of CDK activity. It is thought that Mcm proteins cannot be assembled onto origins while CDKs remain active (Hua *et al.*, 1997; Tanaka *et al.*, 1997; reviewed in Gilbert, 1998; Pasero and Gasser, 1998). It is not known what function the Mcm complex performs, what events are catalysed by CDKs, for what purpose the Cdc7/Dbf4 kinase is also needed, and what step in the initiation process occurs with different kinetics at early- and late-firing origins.

Much of our knowledge about early steps in eukaryotic DNA replication has come from the study of SV40 DNA replication (reviewed in Herendeen and Kelly, 1996). In this system, SV40 T-antigen binds to the replication origin of the viral genome and catalyses unwinding of the origin. Unwinding is viewed as a two-step process. An initial 'opening' by T antigen of the DNA duplex in the immediate vicinity of the origin facilitates binding of replication protein A (RPA). A more extensive region is subsequently stably 'unwound' as a consequence of RPA binding to single-stranded DNA generated by T-antigen helicase activity. RPA and T-antigen then recruit DNA polymerase α (Pol α)-primase, which synthesizes the short RNA sequences needed not only to prime DNA replication on leading strands at origins, but also to prime synthesis of each Okazaki fragment on lagging strands. After synthesizing a short chain of deoxyribonucleotides, Pol α -primase is displaced by replication factor C, which recruits PCNA and DNA polymerase δ (Pol δ). T antigen also acts as a helicase at the replication fork as it moves along the unreplicated DNA duplex. RPA, Pol α -primase, RFC, PCNA and Pol δ also function at the replication fork (reviewed in Stillman, 1994).

In eukaryotic cells, RPA and Pol α -primase consist of three and four subunits, respectively. In yeast, all of the genes encoding these subunits are essential for viability and are suggested to be important for DNA replication initiation (reviewed in Foiani *et al.*, 1997; Wold, 1997). However, it is not known whether RPA and Pol α -primase are recruited to origins in a similar manner to the SV40 system. The cellular proteins that carry out T-antigen

helicase function at chromosomal origins are not known. However, it has recently been reported that a weak helicase activity is associated with a complex composed of Mcm4p, Mcm6p and Mcm7p (Ishimi, 1997). It has also been suggested that during replication elongation Mcm proteins travel along with replication forks (Aparicio *et al.*, 1997), as might be expected if they acted as a helicase.

By immunoprecipitating DNA crosslinked to specific replication factors by formaldehyde, we have detected the origin association of the single-stranded DNA binding protein RPA and DNA primase at the beginning of S phase in *S. cerevisiae*, at least when elongation is prevented or delayed by hydroxyurea (HU). We show that recruitment of Pol α -primase depends upon functional RPA. We also show that association of RPA is dependent on a functional Mcm complex, and regulated by S-CDKs and Dbf4/Cdc7. Finally, we show that early- and late-firing origins differ not in the timing of their recruitment of an Mcm protein but in the timing of the association with origins of RPA, and that this process is regulated by the Rad53 (also known as Sad1, Mec2 and Spk1) protein kinase which was initially identified as a factor essential to arrest cell-cycle in response to DNA damage or replication block (Allen *et al.*, 1994; Weinert *et al.*, 1994; reviewed in Weinert, 1998). We propose that active chromosomal replication origins are formed by the sequential recruitment of ORC, Cdc6p, Mcm complex, RPA and Pol α -primase in this order, and that S-CDKs, Dbf4/Cdc7 and Rad53p are required to properly regulate the recruitment of RPA to origins that have previously assembled the Mcm complex. We postulate that origin unwinding accompanied by RPA association is one of the key steps, if not the only one, that is regulated by S-CDK, Dbf4/Cdc7 and Rad53 protein kinases.

Results

Detecting association of Rfa1p and Pri1p with replication origins

We investigated the association of RPA and DNA primase with replication origins using formaldehyde to crosslink proteins to DNA in intact cells. We prepared extracts from yeast cells treated with formaldehyde, sheared chromatin to an average size of 500 bp and used polymerase chain reaction (PCR) to measure the abundance of specific sequences bound to immunoprecipitated myc-tagged Rfa1p or Pri1p. *RFA1* encodes the p70 subunit of RPA and *PRI1* encodes the p48 subunit of DNA primase (Lucchini *et al.*, 1987; Heyer *et al.*, 1990). We used four sets of PCR primers to amplify ARS1 or ARS305, and three regions surrounding them as controls (Figure 1A). Both origins fire early during S phase (Campbell and Newlon, 1991). We first investigated the association of Rfa1-myc or Pri1-myc with origins in cells either from asynchronous cultures or from synchronous cultures in which small G₁ cells isolated by centrifugal elutriation progressed through the cell cycle. Indirect immunofluorescence demonstrated that both Rfa1-myc and Pri1-myc were localized within nuclei throughout the cell cycle (data not shown). We could not, however, detect any selective precipitation of ARS-containing DNA fragments in either type of culture, presumably because RPA and primase move rapidly with replication forks (at 60 nucleo-

Fig. 1. *In vivo* association of Rfa1 protein with ARS-containing fragments. (A) Genomic intervals around or at ARS1 (and ARS305) amplified by PCR primers. Within the region of chromosome III depicted here, there are no active replication origins except for ARS305 (Campbell and Newlon, 1991). (B) Association of Rfa1-myc with ARS1- (top) and ARS305- (bottom) containing fragments. Early G₁ diploid cells of strain K7141 (*RFA1-myc18*) were isolated by elutriation and then incubated in YEPR (YEPR plus 2% raffinose) in the presence of HU at 25°C. PCR was performed either on immunoprecipitates derived from the same volume of crosslinked cells at each time point or on chromatin fragments from the whole cell extract (WCE) with prior crosslink (at 0 min). The percentage of budded cells is also shown. (C) DNA content was measured by FACS from samples collected in (B) (HU+) and from the same cells incubated in YEPR in the absence of HU at 25°C (HU-). (D) Amplification of ARS1- (top) and ARS305- (bottom) containing fragments in various conditions. PCR was performed either on chromatin fragments isolated after immunoprecipitation procedures (lanes 1–12) or on those from the WCE with prior crosslink (lanes 13–15) or serial 4-fold dilution of WCE (lanes 16 and 17). These samples were prepared when G₁ diploid cells of strain K6700 (*DBF4-myc18*) (lanes 1, 2 and 13), K7141 (*RFA1-myc18*) (lanes 3–8, 14, 16 and 17) or K7274 (*RFA1-myc18*, ARS1/860T→G) (lanes 9–12, 15), reached the indicated budding index in YEPR with HU at 25°C. Control precipitations were performed without prior crosslink (lanes 7 and 8) or without anti-myc antibody (lanes 5 and 6). In the presence of HU, G₁ diploid cells of three strains remained exclusively in 2C DNA contents throughout the progression of budding as in HU+ (C) (data not shown).

Fig. 2. *In vivo* association of Pri1 protein with ARS-containing fragments. (A) Association of Pri1-myc with ARS1- (top) and ARS305- (bottom) containing fragments. G_1 diploid cells of strain K7214 (*PRI1-myc9*) were incubated in YEPR with HU at 25°C and treated as in Figure 1B. In the presence of HU, G_1 diploid cells remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown). (B) Amplification of ARS1- (top) and ARS305- (bottom) containing fragments in various conditions. PCR was performed either on chromatin fragments isolated after immunoprecipitation procedures (lanes 1–10) or on those from the WCE with prior crosslink (lanes 11 and 12). These samples were prepared when G_1 diploid cells of K7214 (*PRI1-myc9*) (lanes 1–6, 11) or K7279 (*PRI1-myc9*, ARS1/860T→G) (lanes 7–10, 12), reached the indicated budding index in YEPR with HU at 25°C. Control precipitations were performed without prior crosslink (lanes 5 and 6) or without anti-myc antibody (lanes 3 and 4). In the presence of HU, G_1 diploid cells of both strains remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown).

tides/s; Rivin and Fangman, 1980) very soon after loading at origins, and because initiation of S phase occurs over a 30 min window in our most synchronous cultures.

To stall fork movement and prolong the period during which RPA and DNA primase might associate with origins, small G_1 cells isolated by elutriation were incubated in the presence of HU, which inhibits ribonucleotide reductase and prevents most of DNA replication (Figure 1C). Under these circumstances, Rfa1-myc associated with ARS1 and ARS305 shortly before the onset of budding, which corresponds to S-phase entry when the same cells were incubated in the absence of HU (Figure 1B and C). Pri1-myc associated with both origins with similar timing (Figure 2A). Preferential amplification of ARS fragments was dependent on the treatment with formaldehyde and on the inclusion of anti-myc antibody. Furthermore, preferential amplification of ARS1 but not ARS305 (from the same DNA preparation) was abolished by a point mutation (ARS1-A) that greatly reduces ARS1 firing (Marahrens and Stillman, 1994) (Figures 1D and 2B). Both Rfa1-myc and Pri1-myc were localized within nuclei throughout the time course (data not shown).

The abundance of ARS-containing fragments in Rfa1p- and Pri1p-specific immunoprecipitates later declined, and in some experiments that of neighbouring fragments increased. This presumably reflects slow movement of replication forks and is consistent with a recent report suggesting that early-firing origins eventually fire in HU (Bousset and Diffley, 1998; Santocanale and Diffley, 1998). We noted that association of Pri1-myc with origins was much more easily detected when small G_1 cells isolated by elutriation were incubated in HU than when larger G_1 cells produced by pheromone treatment were released into HU (data not shown). Replication fork movement in the presence of HU might occur more readily in large cells than in small cells.

We next investigated the inter-dependence of RPA and Pol α -primase association with origins. Association of Pri1-myc with origins was greatly reduced by *rfa2-2*, a temperature-sensitive (ts) mutation of the 34 kDa RPA subunit (Santocanale *et al.*, 1995) or by *cdc17-1*, a ts mutation of the Pol α catalytic subunit (Lucchini *et al.*, 1990, and references therein) (Figure 3). In contrast, association of Rfa1-myc was unaffected by *cdc17-1* (Figure 4A and B). This suggests that association of primase with origins depends on RPA, but that RPA association does not depend on Pol α -primase. These results also suggest that we are able to detect the arrival of RPA at origins in HU even in the absence of appreciable fork movement, which is clearly reduced in *cdc17-1* mutants (compare, for example, the rate at which RPA migrates away from origins in Figure 4A and B). The detection of RPA arriving at origins was not an artifact of the treatment with HU because it could also be detected, albeit for a short time, as G_1 cells of *cdc17-1* mutants (isolated from a culture grown at 25°C) were incubated at 36°C in the absence of HU (data not shown).

Association of RPA with replication origins requires the activity of S-CDKs

Activation of S-CDKs at the G_1 /S boundary is required for the firing of origins that have already recruited Mcm proteins. We therefore investigated whether S-CDKs are required for the association of RPA with origins. We first compared the association of Rfa1-myc with ARS1 and ARS305 in wild-type and *cdc4-1* mutant cells. *CDC4* encodes a WD40 protein needed for degradation of the CDK inhibitor p40Sic1, and *cdc4* mutants arrest without DNA replication in a restrictive temperature because S-CDKs are inhibited by Sic1p (Schwob *et al.*, 1994). We collected small G_1 cells from both wild-type and *cdc4-1* mutant strains and incubated them at 36°C in the presence of HU (Figure 4A and C). Rfa1p associated with both origins in wild-type but not in *cdc4* mutant cells.

Because *CDC4* is required for the degradation of other proteins besides p40Sic1 (reviewed in Krek, 1998), we also analysed cells whose S-CDKs were inhibited by induction from the *GALI* promoter of a non-degradable version of Sic1p (due to mutations of CDK phosphorylation sites T5, T33 and S76 to V5, V33 and A76) (Verma *et al.*, 1997; E.Schwob, T.Böhm and K.Nasmyth, submitted). We collected small G_1 cells from a *GAL-SIC1V5V33A76* strain. In the presence of HU, one half of the population was incubated with glucose (*GALI* promoter off), and the other half with galactose (*GALI* promoter on). The association of Rfa1-myc

Fig. 3. The origin association of PriI protein depends on Rfa2p and Cdc17p (Pol α). Association of PriI-myc with ARS1- (top) and ARS305- (bottom) containing fragments in wild-type (WT) (A), *rfa2-2* (B), or *cdc17-1* (C) cells. G₁ diploid cells of K7214 (*PRII-myc9*), K7360 (*PRII-myc9, rfa2-2*) and K7458 (*PRII-myc9, cdc17-1*) were collected by elutriation from cultures grown at 25°C and incubated in YEPR in the presence of HU at 36°C. PCR was performed as in Figure 1B. DNA content, measured by FACS when cells were incubated in the absence of HU at 36°C, is shown for WT and *rfa2-2* strains (D). In the *cdc17-1* strain (K7458), DNA content was as in *cdc17-1* cells harbouring *RFAI-myc18* (K7140) in the absence of HU at 36°C (see Figure 4F). In the presence of HU, G₁ diploid cells of three strains remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown). ND designates not done.

with both ARS1 and ARS305 was much less in galactose than in glucose (Figure 5A). A few cells probably managed to activate S-CDKs and even complete S phase when incubated in galactose without HU (Figure 5B), which would be consistent with our observation that the association of Rfa1-myc with origins was not completely abolished in galactose with HU (Figure 5A).

Association of RPA with origins depends on Dbf4p and Mcm5p

To investigate the dependence of the origin association of RPA on the Dbf4/Cdc7 protein kinase and on the Mcm

complex, we isolated small G₁ cells from *dbf4-1* and *mcm5* (*cdc46-1*) mutants (Chapman and Johnston, 1989; Hennessy *et al.*, 1990, and references therein) and incubated them at 36°C in the presence of HU (Figure 4D and E). Association of Rfa1-myc with ARS1 was abolished by *dbf4-1* and *mcm5* mutations. Rfa1p association with ARS305 was also abolished by *dbf4-1*, but it was only reduced by *mcm5*. FACS analyses performed on cells incubated at 36°C in the absence of HU showed that DNA replication was abolished by *dbf4-1* but that it was only slowed down by *mcm5* (data not shown and Figure 4F). These data suggest that the *mcm5* mutation affects the loading of RPA at some origins more than at others. We conclude that the loading of Rfa1p onto replication origins depends on a functional Mcm complex and on Dbf4/Cdc7 kinase. We also analysed immunoprecipitates from a synchronous culture of cells carrying a myc-tagged *DBF4* gene but were unable to detect any specific association of Dbf4-myc with either ARS1 or ARS305, either in the presence or the absence of HU (Figure 1D and data not shown).

Mcm7p co-exists with RPA at early-firing origins in HU

To investigate whether Mcm proteins remain associated with origins once they have recruited RPA, we compared the timing of association of Mcm7-myc and Rfa1-myc with ARS1 and ARS305 when G₁ cells isolated by elutriation were incubated in HU (Figures 1B and 6B). Whereas Rfa1p associated with origins when cells budded, Mcm7p associated from the very beginning of G₁ until well after cells had budded. Mcm7p remained associated specifically with ARS sequences as long as Rfa1p did. After the time at which cells should have initiated DNA replication (~1 h), the association of Mcm7p with ARS1 and ARS305 declined like that of Rfa1p and, at least around ARS1, association of Mcm7p with neighbouring fragments increased. We conclude that Mcm7p remains at origins even after RPA has been recruited. Our data are also consistent with the notion that, at least in HU, Mcm proteins move with the replication fork after the origin firing (Aparicio *et al.*, 1997).

Cdc6p and Mcm7p are loaded onto early- and late-firing origins with similar kinetics

To determine whether late-firing origins recruit Cdc6p or Mcm proteins later than early-firing origins, we compared the association of Cdc6p and Mcm7p with ARS1, an early-firing origin, and with ARS501, a late-firing origin (Figure 6A) (Ferguson *et al.*, 1991) as unbudded G₁ cells isolated by elutriation progressed through the cell cycle (in the absence of HU). Both proteins associated with ARS501 as well as ARS1 from the early G₁. The ARS association of Cdc6p declined with similar kinetics at both origins shortly before the onset of S phase (Figure 6C, top), whereas Mcm7p associated until later than Cdc6p and disappeared from the early origin during S phase, ~10–15 min earlier than it disappeared from the late one (Figure 6C, bottom). We conclude that the later firing of ARS501 is not due to a later loading of Mcm proteins. Furthermore our data are consistent with the notion that Mcm proteins leave origins upon replication initiation (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). When cells

Fig. 4. The origin association of Rfa1 protein depends on Cdc4p, Dbf4p and Mcm5p but not on Cdc17p (Pol α). Association of Rfa1-myc with ARS1- (top) and ARS305- (bottom) containing fragments in wild-type (WT) (A), *cdc17-1* (B), *cdc4-1* (C), *dbf4-1* (D) and *mcm5* (*cdc46-1*) (E) cells. G₁ diploid cells of K7141 (*RFA1-myc18*), K7140 (*RFA1-myc18, cdc17-1*), K7276 (*RFA1-myc18, cdc4-1*), K7277 (*RFA1-myc18, dbf4-1*) and K7275 (*RFA1-myc18, cdc46-1*) were collected by elutriation from cultures grown at 25°C and incubated in YEPR in the presence of HU at 36°C. PCR was performed as in Figure 1B. DNA content, measured by FACS when cells were incubated in the absence of HU at 36°C, is shown for WT, *cdc17-1* and *mcm5* strains (F). In *cdc4-1* and *dbf4-1* strains, DNA content was as in *cdc17-1* cells in the absence of HU at 36°C (data not shown). In the presence of HU, G₁ diploid cells of five strains remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown).

were incubated in HU, Mcm7p remained associated with ARS501 throughout the progress of budding, which contrasts with its disappearance from ARS1 and ARS305 ~1 h after bud formation was detected (Figure 6B). This suggests that the delay in replication initiation due to HU is much greater at late-firing origins than at early-firing ones.

RPA associates with early-firing origins but not with a late-firing origin in the presence of HU

In contrast with ARS1 and ARS305 (Figure 1B), Rfa1-myc did not associate with ARS501 when G₁ cells were incubated in the presence of HU (Figure 7, wild-type), even though Mcm7-myc had been recruited to this origin (Figure 6B) and S-CDKs and Dbf4/Cdc7 had probably been activated, at least globally within the cell (Jackson *et al.*, 1993; Stueland *et al.*, 1993). This suggests that early- and late-firing origins differ principally in the timing of recruitment of RPA. Since our assay required inhibition of chain elongation either using HU or a Pol α mutation, we cannot be certain whether this difference also occurs during undisturbed S phases.

Association of RPA with a late-firing origin is regulated by Rad53 kinase

Rad53 encodes a kinase which slows the rate of replication in response to DNA damage (Paulovich and Hartwell, 1995) by preventing initiation from late-firing origins (K.Shirahige and H.Yoshikawa, personal communication). Rad53p also inhibits the firing of late origins during early S phase (K.Shirahige and H.Yoshikawa, personal communication) and in cells treated with HU (Santocanale and Diffley, 1998; see Discussion in Bousset and Diffley, 1998). To test whether Rad53p performs this by blocking the recruitment of RPA to late origins, we analysed the association of Rfa1-myc with an early (ARS305) and a late (ARS501) origin when G₁ cells of a *rad53-21* strain (Sanchez *et al.*, 1996) were incubated in HU. Whereas Rfa1p failed to associate with ARS501 in wild-type cells, Rfa1p associated with ARS501 in *rad53* mutant cells, albeit with a slight delay relative to its association with ARS305 (Figure 7, *rad53-21*). We conclude that the Rad53 kinase prevents recruitment of RPA to a late-firing origin in HU. Rad53p might also be responsible for delaying the firing of late origins during undisturbed S phase by delaying the association of RPA with them.

Fig. 5. The origin association of Rfa1 protein is suppressed when activity of S-CDKs is inhibited. (A) Association of Rfa1-myc with ARS1- (top) and ARS305- (bottom) containing fragments with (Gal) and without (Glc) the expression of non-degradable form of Sic1p (a potent CDK inhibitor) from the *GAL1* promoter. G₁ diploid cells of K7328 (*RFA1-myc18*, *GAL-SIC1V5V33A76*) were incubated in either YEP plus 2% glucose (Glc) or YEPR plus 2% galactose (Gal) in the presence of HU at 25°C. PCR was performed as in Figure 1B. (B) DNA content was measured by FACS when cells were incubated in YEP plus 2% glucose (Glc) or YEPR plus 2% galactose (Gal) in the absence of HU at 25°C. In the presence of HU, G₁ diploid cells remained exclusively in 2C DNA contents in both medium conditions throughout the progression of budding as in Figure 1C, HU+ (data not shown).

Discussion

It has been shown that yeast cells prepare for initiation of DNA replication as they exit from mitosis (Diffley *et al.*, 1994). Potential origins are bound by ORC, which recruits Cdc6p upon its accumulation at telophase and this in turn enables the recruitment of Mcm proteins (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). We show here that the recruitment of Mcm7p occurs with similar if not identical kinetics at origins destined to fire early and late during S phase. Mcm7p association with both early- and late-firing origins is already maximal by the time daughter cells are born. Three different protein kinases regulate steps subsequent to the loading of the Mcm complex. The Dbf4/Cdc7 and S-CDKs are necessary for initiation (reviewed in Diffley, 1996; Nasmyth, 1996; Stillman, 1996), whereas the Rad53 kinase delays initiation at late-firing origins (K. Shirahige and H. Yoshikawa, personal communication). It is unclear how Rad53 and Dbf4/Cdc7 kinases are regulated. On the other hand, S-CDKs are totally inactive during early G₁, and their activation due to accumulation of S-phase cyclins and degradation of the CDK inhibitor p40Sic1 occurs shortly before or simultaneously with the firing of early origins (Schwob *et al.*, 1994). S-CDKs become active long after recruitment of Mcm proteins to origins and must therefore catalyse some subsequent step in the initiation process. CDKs are thought not only to promote initiation from origins that have previously assembled Mcm proteins, but also to inhibit reassociation of the Mcm complex with origins that have already fired and thereby prevent firing of origins more than once per

CDK cycle (Dahmann *et al.*, 1995; Piatti *et al.*, 1996; Hua *et al.*, 1997; Tanaka *et al.*, 1997).

To measure events that occur at origins subsequent to the loading of Mcm proteins, we used a chromatin immunoprecipitation assay to measure the association with origins of Rfa1p, a subunit of the single-stranded DNA binding protein RPA, and Pri1p, a subunit of DNA primase (Lucchini *et al.*, 1987; Heyer *et al.*, 1990). We were unable to detect the specific association of either protein with origins in cells from synchronous or asynchronous cultures, but were able to detect specific association when G₁ cells were cultured in the presence of HU, an inhibitor of ribonucleotide reductase. Treatment with HU greatly slows down or, in very small cells isolated by elutriation, may even completely halt replication fork movement for a short period following bud emergence. We were also able to detect RPA association with origins when fork movement was inhibited by the Pol α mutant *cdc17-1*.

Studies on SV40 replication *in vitro* suggest that the loading of RPA onto origins is catalysed by the initial opening of origin DNA duplex by T antigen (reviewed in Herendeen and Kelly, 1996). RPA loading is therefore a very early step in the initiation process. T-antigen and RPA subsequently move with the replication forks, the former unwinding unreplicated DNA and the latter maintaining it in single-stranded state (reviewed in Stillman, 1994). A key question is whether the ARS-containing fragments that we detect in RPA immunoprecipitates in the presence of HU represents loading of RPA prior to the synthesis of DNA chains or whether it merely represents RPA present at replication forks whose movement

Fig. 6. Association of Mcm7p and Cdc6p with ARS501(a late-firing origin)-containing fragments. (A) Genomic intervals around or at ARS501 amplified by PCR primers. The available data suggest that there are no active replication origins except ARS501 within the region of chromosome V depicted here (Ferguson *et al.*, 1991; Tanaka *et al.*, 1996). (B) Association of Mcm7-myc with ARS1- (top), ARS305- (middle) and ARS501- (bottom) containing fragments. G₁ diploid cells of strain K6465 (*MCM7-myc7*) were incubated in YEPR with HU at 25°C and treated as in Figure 1B. Cells remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown). Cells from asynchronous culture in YEPR in the absence of HU at 25°C (AS) were also analysed. In the right two lanes at the bottom, PCR was performed on chromatin fragments from serial 4-fold dilution of WCE. ARS1-, ARS305- or ARS501-containing fragments were not specifically amplified when immunoprecipitates were prepared from asynchronous culture of K6465 without inclusion of anti-myc antibody or without prior crosslink, or from asynchronous culture of cells without myc-tags (Tanaka *et al.*, 1997; data not shown). (C) Association of Cdc6-myc (top) and Mcm7-myc (bottom) with ARS1- and ARS501- containing regions. G₁ diploid cells of strain K6691 (*CDC6-myc18*) or K6465 were isolated by elutriation and then incubated in YEPR at 25°C in the absence of HU. The previously published data describing association with ARS1 (Tanaka *et al.*, 1997) are presented here for comparison with that with ARS501. For DNA content of cells, see Figure 3 in Tanaka *et al.* (1997).

has been greatly slowed. In the case of the latter, one would expect that inactivation of Pol α should reduce or at least delay RPA association with origin-containing sequences.

As might be expected, inactivation of Pol α due to the *cdc17-1* mutation, reduced Pri1p association with origin-containing fragments (compare Figure 3A with C) and slowed down the transfer of RPA protein from origins to neighbouring sequences (compare Figure 4A with B) in the presence of HU, and greatly reduced bulk DNA

synthesis in the absence of HU (Figure 4F). All these pieces of evidence suggest that fork movement is greatly reduced in *cdc17-1* mutants compared with wild-type cells both in the presence and absence of HU. Nevertheless, the mutation had no effect on the kinetics of RPA association with origin-containing sequences when cells started the bud formation in HU (compare Figure 4A with B). This implies that our assay is able to measure RPA association with origins in the absence of fork assembly, as well as its association with slow-moving replication

Fig. 7. Association of Rfa1 protein with ARS305- (top) and ARS501- (bottom) containing fragments in wild-type (WT) and *rad53-21* mutant cells. G₁ diploid cells of K7141 (*RFA1-myc18*) and K7328 (*RFA1-myc18, rad53-21*) were incubated in YEPR with HU at 25°C. PCR was performed as in Figure 1B. The data of ARS305 association in wild-type cells are the same as in Figure 1B. In the presence of HU, G₁ diploid cells of both strains remained exclusively in 2C DNA contents throughout the progression of budding as in Figure 1C, HU+ (data not shown).

forks. If this is correct, then our observations that Rfa1p association with origins is greatly reduced by inactivation of an Mcm, Dbf4p, or S-CDKs indicate that Dbf4/Cdc7 and S-CDKs are required for RPA loading at origins that have previously assembled the Mcm proteins, and not just for subsequent replication fork movement. If loading of Mcm proteins in the absence of CDKs is the first step towards assembling bi-directional replication forks (Tanaka *et al.*, 1997), then recruitment of RPA promoted by S-CDKs and Dbf4/Cdc7 may be the second step.

Adachi and Laemmli (1994) measured indirectly replication-associated DNA unwinding in *Xenopus* egg extracts. Their measurement was based on the sensitivity of single-stranded chromatin DNA to P1 nuclease digestion and failure to remove RPA from chromatin even with the addition of excess single-stranded DNA. They suggested that DNA unwinding takes place after nuclear membrane formation at the beginning of replication, and that it decreases after treatment with p21Cip1 CDK inhibitor. Their conclusion is consistent with our observation that RPA is recruited to origins at the onset of replication initiation and long after the loading of Mcm proteins, and that RPA recruitment requires S-CDK activity.

Some origins fire early whereas others fire late during S phase. To address what step in the initiation process might be inhibited at 'late-firing' origins, we compared the time of association of an Mcm protein and RPA at early- and late-firing origins. We found that an Mcm protein arrives at both early and late origins when cells exit mitosis, and long before S-CDK activation at the G₁/S boundary. Because we were only able to detect RPA association when fork movement was slowed down, we could not address directly whether RPA arrives at late origins later than early ones in cycling culture. Nevertheless, our observation that RPA is recruited to early origins but not to a late one in the presence of HU, at around the time of S-CDK activation, suggests that this will be the case, since late-origin firing seems to be regulated by a common mechanism (involving Rad53p) both in HU treatment and in cycling culture (see below). We therefore propose that the recruitment of RPA to origins by Mcm proteins, upon S-CDKs and Dbf4/Cdc7 kinase activation, is the step whose timing differs between early- and late-firing origins.

We found the Rad53 protein kinase is required to prevent RPA association with a late-firing origin in the presence of HU. Meanwhile, Santocanale and Diffley (1998) have found that Rad53p also prevents late origin

firing in HU (see Discussion in Bousset and Diffley, 1998). Rad53p is also implicated in arresting anaphase when cells are treated with HU (reviewed in Weinert, 1998) and in preventing the early firing of late origins during undisturbed S phase (K. Shirahige and H. Yoshikawa, personal communication). This suggests that cells monitor either recruitment of RPA or the formation of replication forks at early-firing origins, upon which recruitment of RPA to late-firing origins is blocked by a mechanism involving Rad53p.

There are strong parallels between the roles of SV40 T antigen (reviewed in Herendeen and Kelly, 1996) and those of Mcm proteins. Both associate with origins, are required for recruiting RPA, leave origins when RPA does, and appear to move with replication forks (Aparicio *et al.*, 1997). Furthermore, like T antigen, Mcm proteins possess DNA-dependent ATPase motifs and form a hexameric-ring complex (Adachi *et al.*, 1997). Finally, a weak helicase activity has been found associated with a complex containing Mcm4, Mcm6 and Mcm7 (Ishimi, 1997). A crucial difference between Mcms and T-antigen is the latter's ability to promote origin firing more than once during a single cell cycle. This difference may be due to the assembly of Mcms but not of T-antigen at origins being inhibited by the same CDKs that might promote their enzymatic activity for replication initiation (Hua *et al.*, 1997; Tanaka *et al.*, 1997).

Our results suggest that origin unwinding is a highly regulated aspect of chromosome duplication. Our data do not however exclude the possibility that later steps of replication are also regulated by S-CDKs, Dbf4/Cdc7 or Rad53 protein kinase. They also do not exclude the possibility that earlier (but later than loading of Mcms) events, which may be prerequisites for origin unwinding and RPA loading, are rather direct targets of regulation by these kinases. It has been suggested, for instance, that S-CDKs recruit Cdc45p to chromatin (Zou and Stillman, 1998). S-CDKs might promote origin unwinding by recruiting Cdc45p to origins. Further studies are required to pursue these possibilities, as well as to clarify in more detail the molecular events involved in opening and fully unwinding eukaryotic chromosomal origins.

Materials and methods

All strains, media and methods for flow-cytometric DNA quantitation and centrifugal elutriation were as described previously (Tanaka *et al.*, 1997) unless otherwise stated. HU was used at 0.1 M in culture medium.

RAI1 and *PRII* were tagged at the C-terminus with multiple myc epitopes by homologous recombinations at their original chromosomal loci, using a PCR-based method (W.Zachariae and K.Nasmyth, unpublished). These two tagged proteins were functional *in vivo* because strains grew normally, and these tagged proteins constantly accumulated in nuclei, as far as observed by indirect immunofluorescence, throughout the time course of all experiments shown here. The methods for detection of the *in vivo* association between ARS-containing chromatin fragments and myc-tagged proteins were as described (Tanaka *et al.*, 1997). The sequences of PCR primers used in this assay are available upon request. To compare the amount of origin association of proteins among two or more strains, cells were frozen after the crosslinking procedure and processed for immunoprecipitation together after thawing. Such freezing procedures did not affect the amplification of origin-containing fragments. Proper crosslinking was monitored in each experiment by processing a control sample (from asynchronous culture of *Mcm7-myc7* cells) in parallel with samples of interest.

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