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Mrc1 and Tof1 Regulate DNA Replication Forks in Different Ways during Normal S Phase[D]

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The Mrc1 and Tof1 proteins are conserved throughout evolution, and in budding yeast they are known to associate with the MCM helicase and regulate the progression of DNA replication forks. Previous work has shown that Mrc1 is important for the activation of checkpoint kinases in responses to defects in S phase, but both Mrc1 and Tof1 also regulate the normal process of chromosome replication. Here, we show that these two important factors control the normal progression of DNA replication forks in distinct ways. The rate of progression of DNA replication forks is greatly reduced in the absence of Mrc1 but much less affected by loss of Tof1. In contrast, Tof1 is critical for DNA replication forks to pause at diverse chromosomal sites where nonnucleosomal proteins bind very tightly to DNA, and this role is not shared with Mrc1.

INTRODUCTION

Eukaryotic cells regulate the progression of DNA replication forks in a highly complex manner to preserve genome stability. For example, cells activate checkpoint kinases in response to defects in DNA synthesis or after the encounter of forks with sites of DNA damage, and these kinases play a key role in preserving the integrity of the fork under such conditions (Andreassen *et al*., 2006; Branzei and Foiani, 2006). The progression of DNA replication forks is also regulated in diverse ways during the normal process of chromosome replication, and this allows eukaryotic cells to couple chromosome replication to other cellular processes. For example, pausing of a DNA replication fork at a specific site in the mating type region of fission yeast is linked to the establishment of a genomic imprint (Dalgaard and Klar, 2000). In addition, the progression of replication forks is coupled to the establishment of cohesion between sister chromatids, which allows accurate segregation of the chromosomes during mitosis and also facilitates the repair of double-strand breaks in DNA by homologous recombination (Uhlmann and Nasmyth, 1998; Klein *et al*., 1999; Hanna *et al*., 2001; Sjogren and Nasmyth, 2001; Cortes-Ledesma and Aguilera, 2006).

The progression of eukaryotic DNA replication forks is driven by the heterohexameric DNA helicase MCM2-7 (hereafter MCM), which unwinds the parental DNA duplex and thus allows the replisome machinery to advance (Ishimi, 1997; Labib *et al*., 2000; Pacek and Walter, 2004; Shechter *et al*., 2004; Pacek *et al*., 2006). Work with budding yeast has shown that MCM associates with a large but specific set of regulatory proteins at forks to form so-called "replisome progression complexes" (RPCs) (Gambus *et al*., 2006). In addition to MCM, other components of RPCs include factors such as Cdc45 and the four GINS proteins that are also required for fork progression, and which may play a role in the activation of MCM (Tercero *et al*., 2000; Kanemaki *et al*., 2003; Pacek and Walter, 2004; Pacek *et al*., 2006). Consistent with this view, a complex of Cdc45–MCM–GINS has been isolated from extracts of *Drosophila* embryos, and was found to have an associated DNA helicase activity in vitro (Moyer *et al*., 2006). Moreover, the same proteins have been found to associate with the active helicase at DNA replication forks during replication of a plasmid in extracts of *Xenopus* eggs (Pacek *et al*., 2006).

Other RPC components are not essential to unwind the parental DNA duplex, but instead they are required to regulate fork progression during S phase and thus they maintain genome stability. Among these factors, the proteins known in budding yeast as Mrc1 (mediator of the replication checkpoint) and Tof1 (topoisomerase interacting factor 1) are of particular interest. Mrc1 was originally identified as a factor that is required for checkpoint activation when DNA replication forks stall in response to the depletion of nucleotides (Alcasabas *et al*., 2001). Mrc1 is also needed to restrain progression of the replisome under such conditions, and this role seems to be shared with Tof1 (Katou *et al*., 2003). Moreover, both proteins are important for the recovery of DNA synthesis at stalled forks after the production of nucleotides has been resumed (Tourriere *et al*., 2005). The fission yeast Mrc1 protein and the orthologue of Mrc1 in *Xenopus laevis* (known as Claspin) are also required for checkpoint activation at stalled forks, indicating that this aspect of Mrc1 function has been conserved throughout evolution (Kumagai and Dunphy, 2000; Tanaka and Russell, 2001).

Little is known about the roles of Mrc1 and Tof1 during the normal process of chromosome replication, but it seems

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that both proteins are required to regulate the progression of DNA replication forks. A clue to the function of Tof1 came initially from studies of the orthologous protein in *Schizosaccharomyces pombe*. The fission yeast protein Swi1 is required for a specific DNA replication fork to stop at a "programmed pause site" in the mating type locus (Dalgaard and Klar, 2000). In addition, Swi1 and budding yeast Tof1 are also required for forks to pause at another such site in the rDNA that ensures that replication proceeds in the same direction as the highly active transcription by RNA polymerase I (Krings and Bastia, 2004; Calzada *et al*., 2005; Tourriere *et al*., 2005; Mohanty *et al*., 2006).

Unlike Tof1 and Swi1, the Mrc1 protein is not required for fission yeast cells to switch their mating type, nor is it required in budding yeast for replication forks to pause at the replication fork barrier (RFB) in the rDNA (Calzada *et al*., 2005; Tourriere *et al*., 2005; Mohanty *et al*., 2006). This suggested that Mrc1 might play a different role during normal S phase, and it now seems that Mrc1 is required to maintain the normal rate of progression of DNA replication forks. Two-dimensional DNA gels were used to analyze the progression of individual DNA replication in cells lacking Mrc1, throughout two replicons on chromosomes 3 and 6 of budding yeast (Szyjka *et al*., 2005). This indicated that forks progress very slowly in the absence of Mrc1, and a similar conclusion was reached using chromatin immunoprecipitation (ChIP) of the Cdc45 protein to study progression of the replisome in the presence or absence of Mrc1 (Szyjka *et al*., 2005). We observed a similar phenomenon while using two-dimensional (2D) DNA gels and ChIP to study the progression of DNA replication forks on chromosome 3, but we did not find any evidence for the slow progression of forks in the absence of Tof1 (Calzada *et al*., 2005; data not shown). These findings suggested that Mrc1 and Tof1 might play distinct roles at DNA replication forks during normal S phase, but this view was challenged by a further study that used DNA combing to study the average rate of incorporation of the nucleoside analogue bromodeoxyuridine (BrdU) at DNA replication forks in budding yeast (Tourriere *et al*., 2005). Using this assay, the rate of incorporation of BrdU was found to be equally defective in cells lacking either Mrc1 or Tof1. Cells cannot continue growing after incorporation of BrdU into chromosomal DNA; however, and this approach provides an indirect measure of the kinetics of fork progression. Therefore, it is unclear whether BrdU incorporation reflects the normal rate of progression of unperturbed DNA replication forks. Here, we have used an alternative approach that facilitates a direct and quantitative measure of the normal rate of fork progression in control cells or in cells lacking Mrc1 or Tof1. We have also analyzed the pausing of DNA replication forks at a diverse range of chromosomal loci in the same strains. Our findings indicate that Mrc1 and Tof1 do indeed have different roles at DNA replication forks during the normal process of chromosome replication.

MATERIALS AND METHODS

Yeast Strains and Growth

All budding yeast strains in this study are based on W303-1a (*MAT*a *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3112 can1-100*), and they are listed in Supplemental Table 1. Unless stated otherwise, cells were grown at 24°C in YP medium (1% yeast extract [Difco, Detroit, MI], 2% peptone [Oxoid, Basingstoke, Hampshire, England]) supplemented with 2% glucose (YPD). To synchronize cells in the G1 phase of the cell cycle, α factor mating pheromone was added to a final concentration of 7.5 μg/ml for at least one generation time. Samples for flow cytometry were collected and processed as described previously (Labib *et al*., 1999) and analyzed using FACScan (BD Biosciences, Oxford, United Kingdom).

Two-Dimensional DNA Gels

DNA samples for 2D neutral-neutral gel electrophoresis were prepared and analyzed as described previously (Friedman and Brewer, 1995; Wu and Gilbert, 1995; Lopes *et al*., 2001) (Calzada *et al*., 2005). DNA was digested with the restriction enzyme XbaI.

Gels for the first dimension had an agarose concentration of 0.4%, and they were run for 38 h at 0.7 V/cm. Gels for the second dimension had an agarose concentration of 1%, and they were run for 8 h at 5 V/cm.

The pausing of forks was examined using the following probes (the numbers correspond to budding yeast chromosomal locations according to the *Saccharomyces* Genome Database): *tP(UGG)F* (chromosome 6, 101700-102449), *tA(AGC)F* (chromosome 6, 203751-204738), *CEN3* (chromosome 3, 114786-115385), *CEN4* (chromosome 4, 447981-449273), and *rDNA* (chromosome 12, 466857-467891). Detection was performed using a Personal Molecular Imager FX (Bio-Rad, Hemel Hempstead, United Kingdom).

Dense-Isotope Substitution Experiments

For density-transfer assays, the procedure was essentially as described previously (McCarroll and Fangman, 1988). YHM19 (control), YHM15 (*mrc1*Δ), and YHM17 (*tof1*Δ) cells were grown at 24°C for seven generations in heavy minimal medium containing 0.1% [^{13}C]glucose and 0.01% [^{15}N]$(NH_4)_2SO_4$ (CK Gas Products, Hook, Hampshire, United Kingdom) as carbon and nitrogen source, respectively. Cells were then arrested in G1 phase with mating pheromone as described above, and then they were transferred to YPD medium (predominantly containing the light isotopes ^{12}C and ^{14}N) for 1 h in the continued presence of mating pheromone. The cells were spun, washed twice with YPD medium, and then released from G1 arrest at 24°C in the absence of mating pheromone. Samples were taken for analysis between 20 and 60 min. Chromosomal DNA was extracted and digested with ClaI, and then it was separated on CsCl gradients (McCarroll and Fangman, 1988). The gradients were fractionated subsequently, and every second fraction between refractive indices of 1.407 and 1.403 was transferred to a "slot-blot" apparatus, applied to Hybond-N+ membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and hybridized with specific [α^{32}P]dCTP-labeled probes (see below). A Storm 860 PhosphorImager (GE Healthcare) was used for quantification of the resultant signals in each peak. The extent of replication for each fragment was calculated using the equation % replication = 100 [0.5 HL/(HH + 0.5 HL)], where HL is the area under the HL peak and HH is the area under the HH peak. The specific probes for the various sites on chromosomes 3 and 6 correspond to the following loci (the numbers correspond to budding yeast chromosomal locations according to the *Saccharomyces* Genome Database): *ARS306* (chromosome 3, 73001-73958), *ARS306 + 10kb* (chromosome 3, 61738-62737), *ARS306 + 29kb* (chromosome 3, 45020-46048), *ARS306 + 52kb* (chromosome 3, 21295-22320), *ARS306 + 72kb* (chromosome 3, 2062-3100), *ARS607* (198946-199833), *ARS607 + 19kb* (218567-219216), *ARS607 + 36kb* (233370-234269), and *ARS607 + 62kb* (260047-261087).

The rate of fork progression throughout the 29-kb region to the left of *ARS306* was calculated as follows: the time at which each sequence to the left of *ARS306* was replicated in 20% of cells was determined from the graphs shown in Figures 1B, 2B, and 3B, and then it was plotted against the distance of that site from *ARS306*. The slope of the resultant graph was then determined, and this slope corresponded to the rate of fork progression.

RESULTS

Mrc1 but Not Tof1 Is Crucial for the Normal Rate of Fork Progression

To determine the rate of fork progression during normal S phase in a direct and quantitative manner, in cells lacking either Mrc1 or Tof1, we used dense-isotope substitution. We used strains lacking the origins *ARS305*, *ARS608*, and *ARS609*, so that we could study fork progression at various points throughout two replicons that were each about 70 kb, between *ARS306* and the left end of chromosome 3, and between *ARS607* and the right end of chromosome 6 (Figure 1). We grew wild-type cells for seven generations at 24°C in media containing ^{13}C and ^{15}N (which are natural nonradioactive isotopes of carbon and nitrogen that do not perturb cell growth) so that the chromosomal DNA was fully labeled. We then synchronized cells in G1 phase with mating pheromone, released them in fresh medium containing the light isotopes of carbon and nitrogen ^{12}C and ^{14}N, and we took samples every 10 min between 20 and 60 min. As shown in Supplemental Figure 1, chromosome replication occurred during the time course of the experiment. We then extracted chromosomal DNA from each sample, digested it

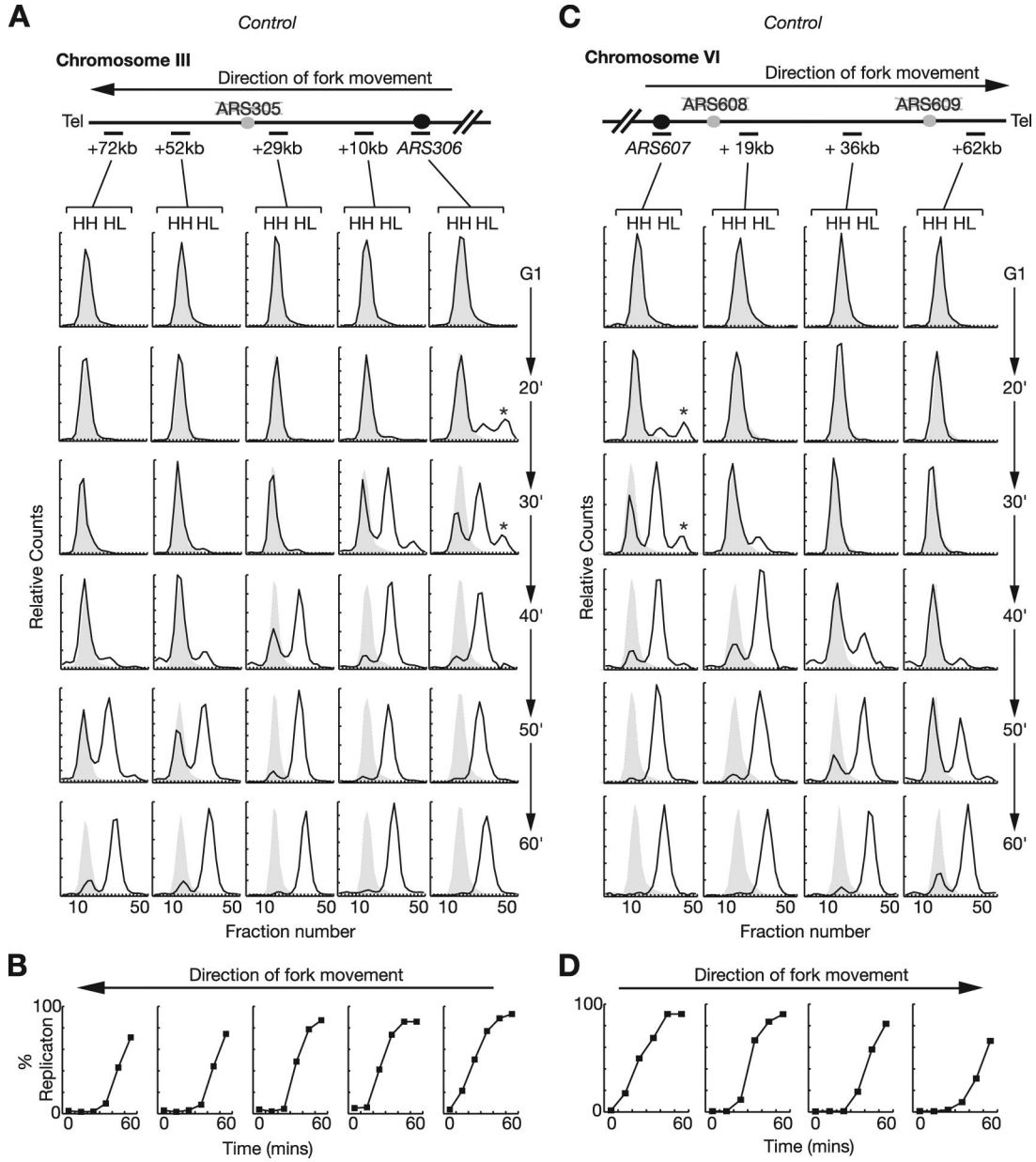

Figure 1. The progression of DNA replication forks on chromosomes 3 and 6 in control cells during normal S phase. The chromosomal origins *ARS305*, *ARS608*, and *ARS609* were deleted in the strains used in this work. (A and B) Progression of the leftward fork from *ARS306* to the left end of chromosome 3. (C and D) Progression of the rightward fork from *ARS607* to the right end of chromosome 6. Semiconservative replication of each site causes the corresponding restriction fragment to move from the heavy-heavy peak (HH) in the cesium chloride gradient to the heavy-light (HL) peak. The asterisk marks a less-dense peak that occurs transiently at the origins as they are replicated. This peak can also be detected 10 kb to the left of *ARS306*, but it is not seen further away from the origins, perhaps due to loss of synchrony as forks progress away from the site of initiation. We do not know at present whether the less-dense peak represents replication intermediates themselves (bubbles and forks) that are less dense than unbranched double-stranded DNA due to their structure, or whether it is produced by the annealing of the newly synthesized "light" daughter strands at replication forks.

with a restriction enzyme, and then ran the samples on cesium chloride gradients. Samples from the gradients were then fractionated and used to generate slot-blots that were hybridized with probes corresponding to specific chromosomal loci on chromosomes 3 and 6. Before replication, each fragment of DNA was present at a point in the cesium chloride gradient that corresponded to fully substituted HH DNA. After one round of semiconservative DNA replication, each fragment became less dense, and so it moved to a new position in the gradient, corresponding to HL DNA. Interestingly, a third population of DNA molecules is always observed transiently at origins during S phase in such experiments (Figure 1, A and C, peak marked by an asterisk). The appearance of this transient population of less dense molecules correlates with the presence of replication intermediates in the cell, and the peak is harder to observe away from origins, perhaps due to the rapid loss of synchrony as forks move away from the site where replication began. As shown in Figure 1, A and C, replication of *ARS306* and *ARS607* was first detected 20 min after release from G1

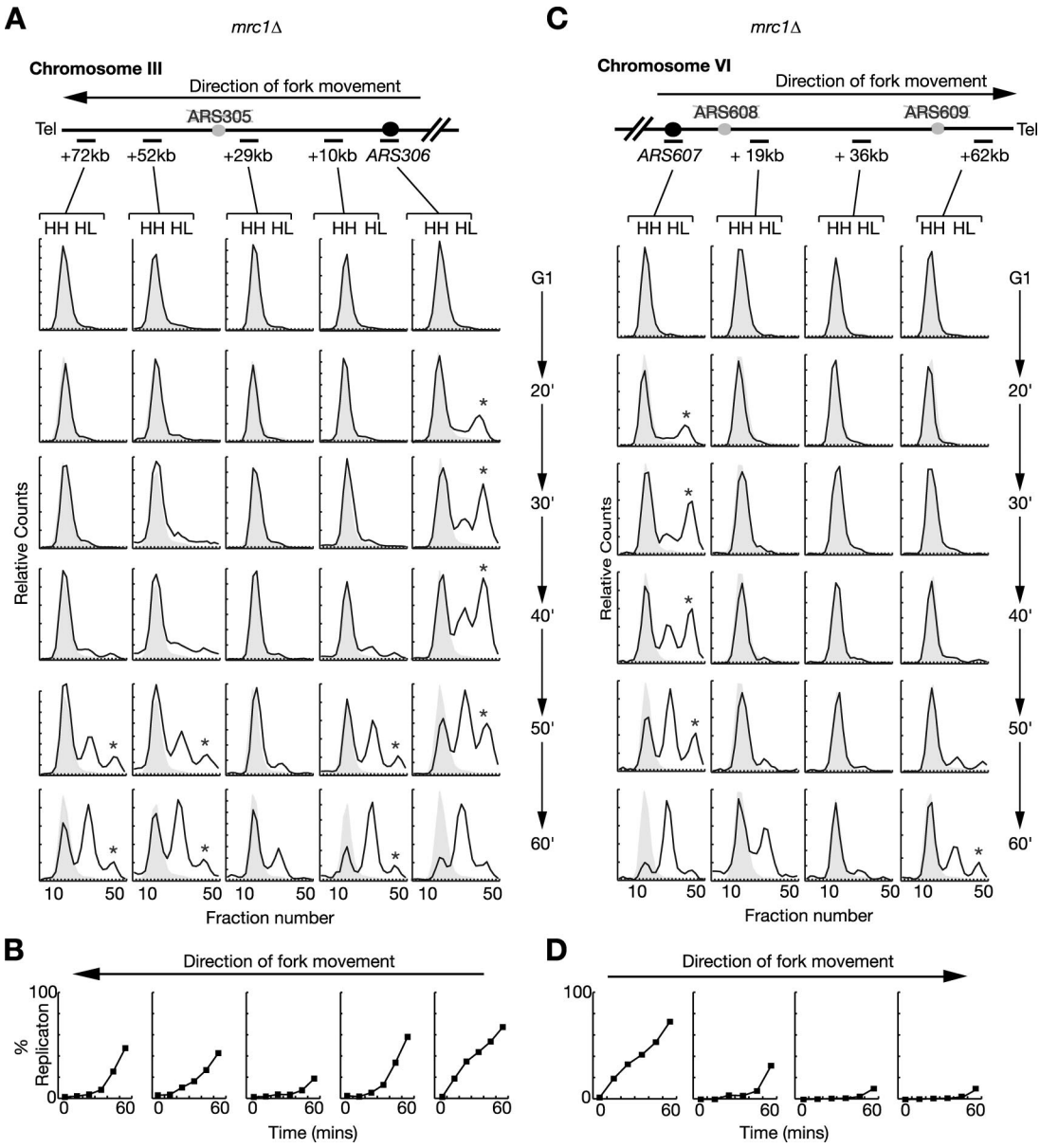

Figure 2. DNA replication forks progress very slowly in the absence of Mrc1. The layout and annotation are the same as in Figure 1.

arrest, and both origins were replicated in most cells by 40 min and in almost all cells by 50 min. The fork to the left of *ARS306* and the fork to the right of *ARS607* moved through the corresponding regions during the course of the experiment, and by 60 min both replicons were completely replicated in almost all cells. We quantified the data, as shown in Figure 1, B and D, and the data for chromosome 3 were used to calculate the rate of fork progression, as described in *Materials and Methods*. This indicated that the leftward fork from *ARS306* moved at 1.9 kb/min under these conditions (Supplemental Table 2).

We then performed an analogous experiment using cells lacking Mrc1. The completion of chromosome replication was slightly slower in this strain (Supplemental Figure 1), consistent with previous findings (Alcasabas *et al.*, 2001; Szyjka *et al.*, 2005), but initiation of replication at *ARS306* and *ARS607* was first detected at 20 min just as in the control strain (Figure 2, A and C). Strikingly, however, the progression of replication forks throughout the two replicons was much slower in the *mrc1*Δ strain. In wild-type cells, the transiently observed less-dense peak at *ARS306* and *ARS607* normally represents a small proportion of the DNA molecules during the period of replication of these origins. This proportion was greatly increased in the absence of Mrc1 (Figure 2, A and C), indicating that replication of the fragments containing the two origins was completed much more slowly. This was confirmed by the slower appearance of the HL peak, such that replication was still incomplete even after 50 min in many cells. As expected, further progression of the leftward fork from *ARS306* and the rightward fork from *ARS607* was also much slower than in the control strain. Previous work has shown that the delayed arrival of forks from earlier origins allows normally silent origins to become active, including the silent origins *ARS300-304* on chromosome 3, and the normally silent origins associated with telomeres (Santocanale *et al.*, 1999; Vujcic *et al.*, 1999).

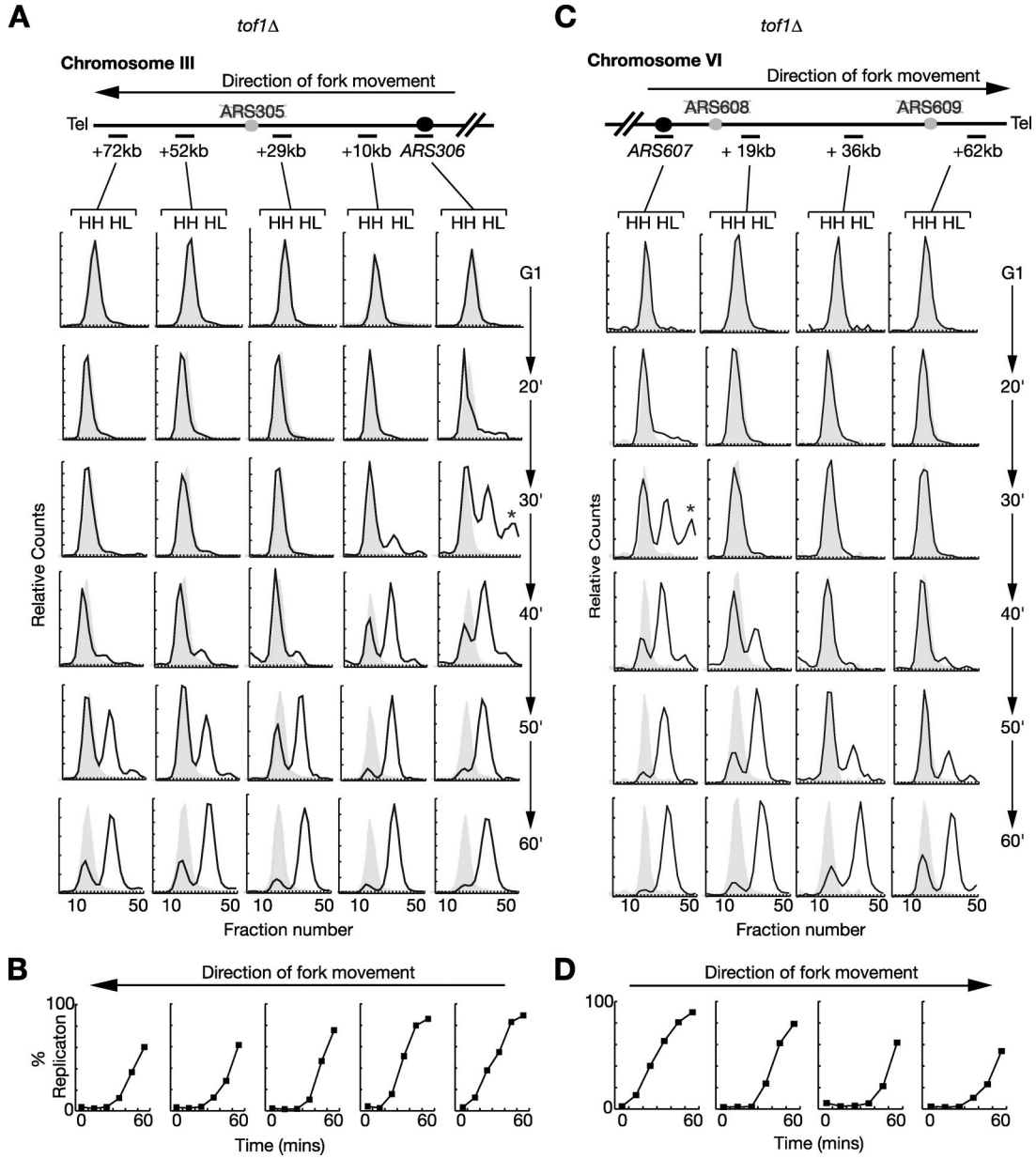

Figure 3. The rate of progression of DNA replication forks in the absence of Tof1 is more similar to control cells than to cells lacking Mrc1. The layout and annotation are the same as in Figure 1.

Consistent with this fact, the sites 52 and 72 kb to the left of *ARS306* were replicated to a greater extent than the site 29 kb from *ARS306* (Figure 2, A and B), indicating the contribution of forks from newly activated origins to the left of the 29-kb fragment (probably the *ARS300-304* cluster). Similarly, the fragment near the end of chromosome 6 was replicated in some cells (probably due to activation of a subtelomeric origin), even though the fragment 36 kb from *ARS607* remained unreplicated in almost all cells throughout the course of the whole experiment (Figure 2, C and D). These data indicate that the two forks established at *ARS306* and *ARS607* move very slowly away from the origins in the absence of Mrc1, and then they continue to progress slowly throughout the two replicons. The activation of new origins on chromosome 3 complicated the calculation of fork rate, but the data for the first 29 kb to the left of *ARS306* on chromosome 3 allowed us to calculate that the leftward fork from this origin moved at a speed of 0.8 kb/min in cells lacking Mrc1 (see *Materials and Methods*). This corresponds to 42% of the speed of the equivalent fork in wild-type cells (Supplemental Table 2), in agreement with the findings of a previous study (Szyjka *et al*., 2005).

Finally, we performed a similar experiment using cells that lacked Tof1 (Figure 3). The first signs of replication at *ARS306* and *ARS607* were again detected after 20 min, but forks then moved away from each origin in a manner that was much more similar to the control than to cells lacking Mrc1. This was apparent in four aspects of the data. First, the transient less-dense peak at *ARS306* and *ARS607* only represented a small proportion of the molecules during the period of time within which the origins replicated. Second, replication of the two origins was completed in almost all

cells by 50 min. Third, replication throughout the two regions of study was only seen to proceed in a leftward manner from *ARS306*, and in a rightward manner from *ARS607*, indicating that fork progression throughout the two replicons was fast enough to prevent activation of normally silent origins. Finally, DNA replication forks reached the end of both replicons in the majority of cells by the end of the experiment. The data were quantified and used to show that the leftward fork from *ARS306* moved at a rate of 1.5 kb/min (83% of the speed of the fork in wild-type cells; Supplemental Table 2). These experiments indicate that Mrc1 is much more important than Tof1 for determining the normal rate of fork progression.

Tof1 but Not Mrc1 Mediates Pausing of Forks at Diverse Protein–DNA Barriers

Tof1 is required for the pausing of DNA replication forks at the RFB in the rDNA, but the same is not true of Mrc1. We wanted to determine whether this difference reflects a general role for Tof1 in the pausing of DNA replication forks at protein–DNA barriers, which is not shared with Mrc1. One previous study reported that Tof1 is required for forks to pause at a specific point on a plasmid that was proposed to represent a collision site with the RNA polymerase III transcriptional machinery coming in the opposite direction from a tRNA (Mohanty *et al*., 2006). Previous work has shown that the promoters of endogenous tRNAs in budding yeast chromosomes represent unidirectional barriers to the progression of DNA replication forks (Deshpande and Newlon, 1996). There is some evidence to indicate that such barriers function independently of transcription, suggesting that pausing of a fork might result from the encounter with tightly bound protein–DNA complexes at the tRNA promoter (Ivessa *et al*., 2003). To test whether Tof1 is required for the accidental pausing of forks at unidirectional replication fork barriers in budding yeast chromosomes, we used 2D DNA gels to examine fork progression at two endogenous tRNAs on chromosome 6. Control and *tof1*Δ cells were grown at 24°C, synchronized in G1 phase, and then released into S phase, after which samples were taken every 15 min (Figure 4A). The pausing of DNA replication forks at *tP(UGG)F* and *tA(AGC)F* was observed in the control strain as a discrete spot at the corresponding position on the Y-arc (Figure 4, B and C, control). However, in the absence of Tof1, the pause site was lost, showing that Tof1 is normally required for forks to pause at this class of accidental RFB on budding yeast chromosomes (Figure 4, B and C, *tof1*Δ). We then examined cells lacking Mrc1, and we saw that the pausing of forks at both sites did not require Mrc1 (Figure 4, B and C, *mrc1*Δ), consistent with previous findings regarding other tRNAs on chromosome 3 (Szyjka *et al*., 2005).

DNA replication forks are also known to pause upon encountering the protein–DNA complexes that are associated with centromeres (Greenfeder and Newlon, 1992). These represent a distinct class of accidental replication fork barrier, because they are able to pause the progression of forks that arrive from either direction. We therefore examined whether Tof1 and Mrc1 are required for forks to pause at such bidirectional barriers. We stripped the membranes from the previous experiments, and then we rehybridized using probes specific for *CEN3* or *CEN4*. As shown in Figure 5, forks paused at both centromeres in the control strain, but pausing was lost in the absence of Tof1 (Figure 5, A and B, *tof1*Δ). In contrast, forks were still able to pause at both *CEN3* and *CEN4* in the absence of Mrc1 (Figure 5, A and B, *mrc1*Δ). Finally, we stripped and rehybridized the same membranes with a probe that was specific for the rDNA, as

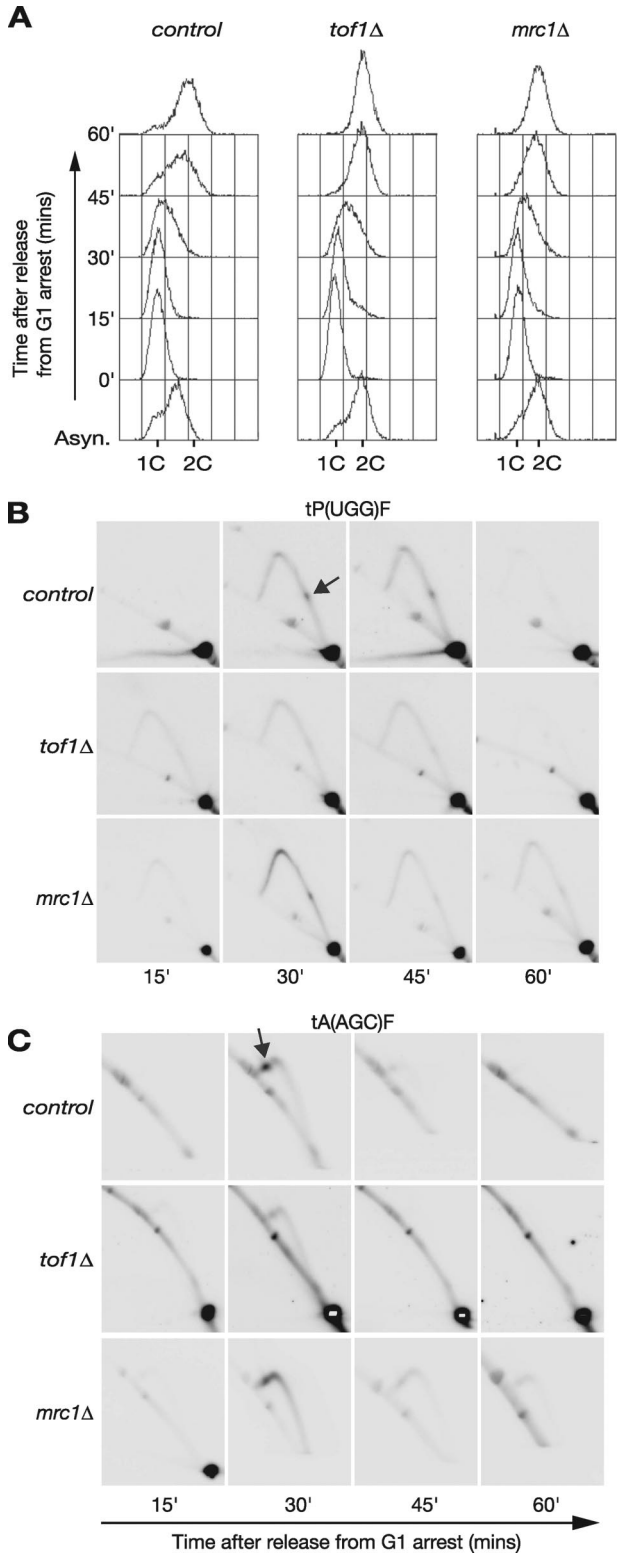

Figure 4. Tof1 but not Mrc1 is required for DNA replication forks to pause at chromosomal sites that correspond to tRNAs. (A) The indicated strains were released from G1 arrest at 24°C in YPD medium, and samples were taken every 15 min. DNA content was measured by flow cytometry. (B and C) Two-dimensional DNA gels were used to detect replication intermediates at sites on chromosome 6 that correspond to the tRNAs tP(UGG)F and tA(AGC)F.

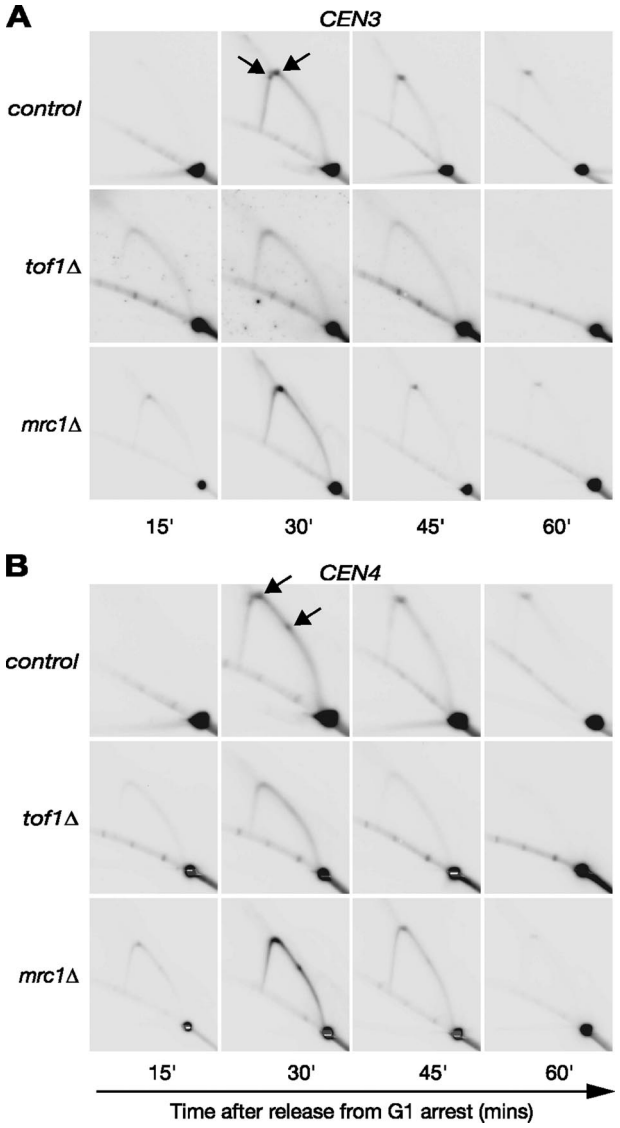

Figure 5. The pausing of DNA replication forks at centromeres requires Tof1 but not Mrc1. (A and B) Two-dimensional DNA gels were used to detect replication intermediates at the centromeres of chromosomes 3 and 4.

a control to confirm our previous observation that forks are indeed able to pause at the RFB in the rDNA in wild-type and *mrc1*Δ strains, but that they pause very inefficiently at this site in cells lacking Tof1 (Supplemental Figure 2).

These experiments show that Tof1 is required for DNA replication forks to pause at diverse protein–DNA barriers in budding yeast. These include a programmed pause site (rDNA RFB) as well as accidental barriers, regardless of whether the latter are unidirectional (tRNA) or bidirectional (centromeres). In contrast, the Mrc1 protein is not required for DNA replication forks to pause at such protein–DNA barriers.

DISCUSSION

The technique of dense-isotope substitution allows a quantitative and kinetic analysis of the progression of individual DNA replication forks, and it does not require the incorporation into chromosomal DNA of agents such as BrdU that cannot support cell growth. Our data indicate that Mrc1 but not Tof1 is crucial for the normal rate of progression of DNA replication forks in budding yeast.

These findings contrast with those of a previous study that used "combing" of chromosomal DNA fibers to show that DNA replication forks have a reduced ability to incorporate BrdU in budding yeast cells lacking either Tof1 or Mrc1 (Tourriere *et al*., 2005). Interestingly, a very recent study has shown that depletion of the Timeless protein, which is probably the human orthologue of Tof1, also causes a similar defect in the incorporation of BrdU at DNA replication forks in human cells (Unsal-Kacmaz *et al*., 2007). Therefore, these experiments support each other, and the data were used to argue that budding yeast Tof1 and human Tim1 are important to determine the normal rate of progression of DNA replication forks during S phase. Although these findings are clearly very interesting, our data indicate—at least for budding yeast—that such experiments may not reflect the normal rate of progression of forks during S phase in the absence of Tof1. It is possible that the progression of DNA replication forks lacking Tof1 might be particularly sensitive to the incorporation of BrdU. Alternatively, the reduction in BrdU incorporation in the absence of Tof1 could perhaps reflect other events that occur in the presence of the nucleoside analogue, such as an increased rate of fork collapse, rather than a slower rate of progression per se. Either way, our data involving noninvasive measures of fork progression indicate that Mrc1 is critically important for determining the normal rate of progression of DNA replication forks in comparison with Tof1.

It remains to be determined how Mrc1 governs the rate of fork progression, but it is interesting to note that it forms part of replisome progression complexes that are built at nascent forks around the MCM helicase (Katou *et al*., 2003; Nedelcheva *et al*., 2005; Gambus *et al*., 2006). In prokaryotes, there are several examples of optimal fork rate being promoted by a physical association between the replicative helicase and the DNA polymerase that is responsible for synthesis of the leading-strand (Dong *et al*., 1996; Kim *et al*., 1996; Yuzhakov *et al*., 1996). By coupling unwinding of the DNA duplex to ongoing DNA synthesis, it seems that the helicase might be prevented from slipping backward, so that forks advance more rapidly (Stano *et al*., 2005). It will be very interesting in future studies to determine whether specific RPC components such as Mrc1 couple the MCM helicase to the eukaryotic leading-strand polymerase either directly or indirectly.

In contrast to Mrc1, Tof1 is crucial for the pausing of DNA replication forks at diverse protein–DNA barriers. The relative roles of the orthologues of Mrc1 and Tof1 in the pausing of DNA replication forks at protein–DNA barriers have not been examined directly in other eukaryotic species. But it is clear that the fission yeast homologue of Tof1 is required for forks to pause at programmed pause sites in the mating type locus and in the rDNA (Dalgaard and Klar, 2000; Krings and Bastia, 2004). The mechanism that underlies the role of Tof1 in pausing remains unclear, although, once again, it is interesting to note that Tof1 is a component of RPCs that are built around MCM (Gambus *et al*., 2006). The budding yeast Tof1 protein was first identified through a two-hybrid screen for factors that interact with DNA topoisomerase 1 (Park and Sternglanz, 1999), but the significance of this apparent interaction remains unclear. It is tempting to speculate that Tof1 forms part of a "molecular brake" that is able to inhibit progression of the helicase when the fork encounters nonnucleosomal proteins that are very tightly bound to DNA.

Two very recent studies also support the idea that Mrc1 and Tof1 play distinct roles during the normal process of chromosome replication in budding yeast. Cells lacking either Mrc1 or Tof1 have a mild defect in the establishment of cohesion between sister chromatids during S phase, but the absence of both proteins causes an additive defect, indicating that Mrc1 and Tof1 contribute to the establishment of cohesion in different ways (Xu *et al*., 2007). In addition, cells lacking Mrc1 have shorter telomeres than control cells, but absence of the Tof1 protein does not affect the length of telomeres, again indicating that the proteins act differently (Grandin and Charbonneau, 2007).

It is clear that both Mrc1 and Tof1 are important for the maintenance of genome stability (Tourriere *et al*., 2005; Admire *et al*., 2006; Chin *et al*., 2006; Robert *et al*., 2006), and it now seems from work in budding yeast that the progression of DNA replication forks is regulated in a highly sophisticated manner, both during normal S phase and in response to DNA damage or other problems in DNA synthesis. Each of the proteins that is associated with the MCM helicase at budding yeast replication forks has a single orthologue in all eukaryotic species, and it thus seems likely that the mechanisms regulating the progression of DNA replication forks will have been very highly conserved throughout the course of evolution.

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