doi:10.1016/j.jmb.2005.01.041 J. Mol. Biol. (2005) 347, 509–521

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Uncoupling of Unwinding from DNA Synthesis Implies Regulation of MCM Helicase by Tof1/Mrc1/Csm3 Checkpoint Complex

Marina N. Nedelcheva[1], Assen Roguev[2], Luben B. Dolapchiev[1] Andrej Shevchenko[3], Hristo B. Taskov[4], Anna Shevchenko[3] A. Francis Stewart[2] and Stoyno S. Stoynov[1,2]*

[1]*Institute of Molecular Biology Bulgarian Academy of Sciences Sofia 1113, Bulgaria*

[2]*Genomics, BioInnovation Zentrum, University of Technology Dresden, Tatzberg 47-51, 01307 Dresden, Germany*

[3]*Max-Planck Institute for Cell Biology and Genetics Pfotenhauerstr. 108, Dresden 01307 Germany*

[4]*National Center of Infectious and Parasitic Diseases, Sofia 1504, Bulgaria*

**Corresponding author*

The replicative DNA helicases can unwind DNA in the absence of polymerase activity *in vitro*. In contrast, replicative unwinding is coupled with DNA synthesis *in vivo*. The temperature-sensitive yeast polymerase α/primase mutants *cdc17-1*, *pri2-1* and *pri1-m4*, which fail to execute the early step of DNA replication, have been used to investigate the interaction between replicative unwinding and DNA synthesis *in vivo*. We report that some of the plasmid molecules in these mutant strains became extensively negatively supercoiled when DNA synthesis is prevented. In contrast, additional negative supercoiling was not detected during formation of DNA initiation complex or hydroxyurea replication fork arrest. Together, these results indicate that the extensive negative supercoiling of DNA is a result of replicative unwinding, which is not followed by DNA synthesis. The limited number of unwound plasmid molecules and synthetic lethality of polymerase α or primase with checkpoint mutants suggest a checkpoint regulation of the replicative unwinding. In concordance with this suggestion, we found that the Tof1/Csm3/Mrc1 checkpoint complex interacts directly with the MCM helicase during both replication fork progression and when the replication fork is stalled.

Keywords: DNA unwinding; DNA replication; supercoiling; replication checkpoint; MCM complex

Introduction

The *de novo* synthesis of DNA in *Saccharomyces cerevisiae* is initiated by a four-subunit polymerase α/primase complex.[1] The 48 kDa and 58 kDa primase subunits lead the synthesis of an RNA primer, which is then utilized by the 180 kDa subunit of polymerase α to synthesize the first DNA nucleotides. The fourth 86 kDa subunit appears to have no enzymatic function but is involved in the regulation of the replication reaction.[2] For the initiation of replication, the polymerase α/primase complex requires single-stranded DNA. The unwinding of the origin and the loading of polymerase α/primase complex at the leading strand starting point require several events.[3] The origin recognition complex (ORC), which is bound to the origin of replication throughout the cell-cycle,[4] is required for the association of Cdc6, Cdt1 and the six minichromosome maintenance (MCM) proteins to the chromatin at the end of the M phase and the beginning of the G1 phase to form a prereplication complex (pre-RC).[5–9] For this assembly to take place, Cdc28/B cyclin must be inactivated.[10] Cdc45 and Sld3 proteins bind to chromatin at late G1 phase and the pre-RC transforms into a preinitiation complex (pre-IC).[6,11,12] The activation of Cdc28/B and the Cdc7/Dbf4 protein kinases[10] trigger this complex to unwind the DNA origin and recruit the polymerase α/primase.[13] This step requires replication protein A (RPA) and the Dpb11–Sld2 complex.[14–16] Finally, polymerase α/primase initiates leading strand

Abbreviations used: ORC, origin recognition complex; MCM complex, minichromosome maintenance complex; pre-RC, prereplication complex; pre-IC, preinitiation complex; HU, hydroxyurea; FACS, fluorescence-activated cell sorter; TAP, tandem affinity purification; MMS, methylmethane sulphonate.

E-mail address of the corresponding author: stoynov@obzor.bio21.bas.bg

DNA synthesis from a single point, adjacent to the binding site for the ORC.[17] The synthesized RNA/DNA primer is then elongated by the processive polymerases δ and ε. The elongation of DNA replication requires unwinding of DNA, intact MCM complex and Cdc45 protein.[6,18,19] When DNA is damaged or DNA replication is blocked, *S. cerevisiae* cells induce Mec1/Rad53-dependent DNA damage and replication checkpoints, which slow DNA replication, stabilise replication forks, induce inhibition of late origin firing and delay cell-cycle progression.[20–23]

Replicative unwinding and DNA synthesis are normally coupled during DNA replication *in vivo*. It has been demonstrated that the DNA unwinding, which is not followed by DNA synthesis, provokes additional negative supercoiling of plasmid molecules during simian virus 40 (SV40) T antigen-dependent replication *in vitro*.[24] During the replication of plasmid DNA in *Xenopus* egg extract, a highly supercoiled presynthetic intermediate was observed.[25] Negative supercoiling in these experiments is a final result of the removal (by the topoisomerases) of the unrestrained compensatory positive supercoiling, generated from the DNA unwinding. When polymerase α or primase was inhibited in the *Xenopus* extract, the negative supercoiling was increased significantly. Thus, it appears that replicative DNA helicases can become uncoupled from the replication fork *in vitro*.

In contrast to the *in vitro Xenopus* egg extract experiments, additional replicative negative supercoiling has not so far been visualised *in vivo*. Extra negative supercoiling was not detected in the thymidylate kinase *cdc8-1* yeast mutant, which displays a quick stop replication arrest when shifted to a restrictive temperature.[26] This lack of additional negative supercoiling could be explained in several ways: DNA is not unwound extensively when DNA synthesis is prevented; DNA unwinding is balanced by changes in the protein DNA architecture or a short DNA synthesis masks DNA unwinding during the *cdc8-1* mutant arrest (the partially replicated regions take positions that are closer to the starts in chloroquine/agarose gel electrophoresis).

To investigate the interaction between the unwinding and synthesis of DNA *in vivo* we examined the changes in the supercoiling of *S. cerevisiae* plasmids during DNA replication, using several temperature-sensitive mutant strains. We report that some plasmid molecules in polymerase α/primase mutant strains became extensively negatively supercoiled during the G_1–S phase of the cell-cycle. In contrast, additional negative supercoiling was not detected in *mcm2-1*, *cdc45-1* or *cdc7-4* mutants, deficient in initiation of DNA replication. Altogether, these results indicate that the extensive negative supercoiling of DNA is a result of replicative unwinding, which is not followed by DNA synthesis. The detection of massive unwinding suggests strongly that when cells are deficient in polymerase α/primase activity, the helicase function can uncouple from DNA synthesis *in vivo*. The limited number of unwound plasmid molecules and synthetic lethality of polymerase α or primase mutants with checkpoint proteins suggests a checkpoint regulation of the replicative unwinding. We show that Tof1/Csm3/Mrc1 checkpoint complex interacts directly with the MCM helicase during both replication fork progression and when the replication fork is stalled.

Results

Analysis of the rate of supercoiling of the 2 μm plasmid throughout the progression of the cell-cycle

The 2 μm yeast plasmid is a convenient object for measuring alterations in DNA topology *in vivo*. The DNA replication of this plasmid is initiated at a single origin[27,28] under the control of the cell-cycle.[29] It has been shown that the 2 μm plasmid, isolated from exponentially growing *S. cerevisiae* cells, has a typical topoisomer distribution pattern.[30,31] To examine the topological changes of DNA, during replication *in vivo*, the rate of supercoiling of the 2 μm plasmid throughout the progression of the cell-cycle was analysed. Yeast cells, bearing the *cdc17-1* temperature-sensitive mutation[32] were incubated at a permissive temperature (under such conditions there is a normal polymerase α activity). Cells were synchronized by α pheromone. DNA was isolated at different time-points after release from the block. The topology of the 2 μm DNA was analysed by chloroquine/agarose gel electrophoresis. This technique[33] resolves negatively supercoiled plasmid DNA into a distribution of topoisomers, which reflects the heterogeneity of plasmid DNA topology *in vivo*. Southern blotting, using a 2 μm plasmid-specific probe, revealed series of bands representing topoisomers differing in linking number by steps of 1. No significant changes in the supercoiling of the 2 μm plasmid were detected throughout the cell-cycle (Figure 1(a)). The same results were obtained using wild-type yeast cells (Figure 1(c)).

To reveal the pattern of distribution of the topoisomers according to their rate of negative supercoiling, we carried out a two-dimensional chloroquine/agarose gel electrophoresis for the 36th minute probe (from the beginning of S phase) of the wild-type culture, after release from the blocking agent. This technique permits separation of topoisomers that differ widely in linking number.[31] It results in a distribution of topoisomers that forms an arc, in which negative superhelicity increases counterclockwise around the arc. Figure 1(d) shows that the fastest-migrating topoisomers on one-dimensional electrophoresis are positioned in the left part of the arc and are those with the highest negative supercoiling.

It was not surprising that we did not detect

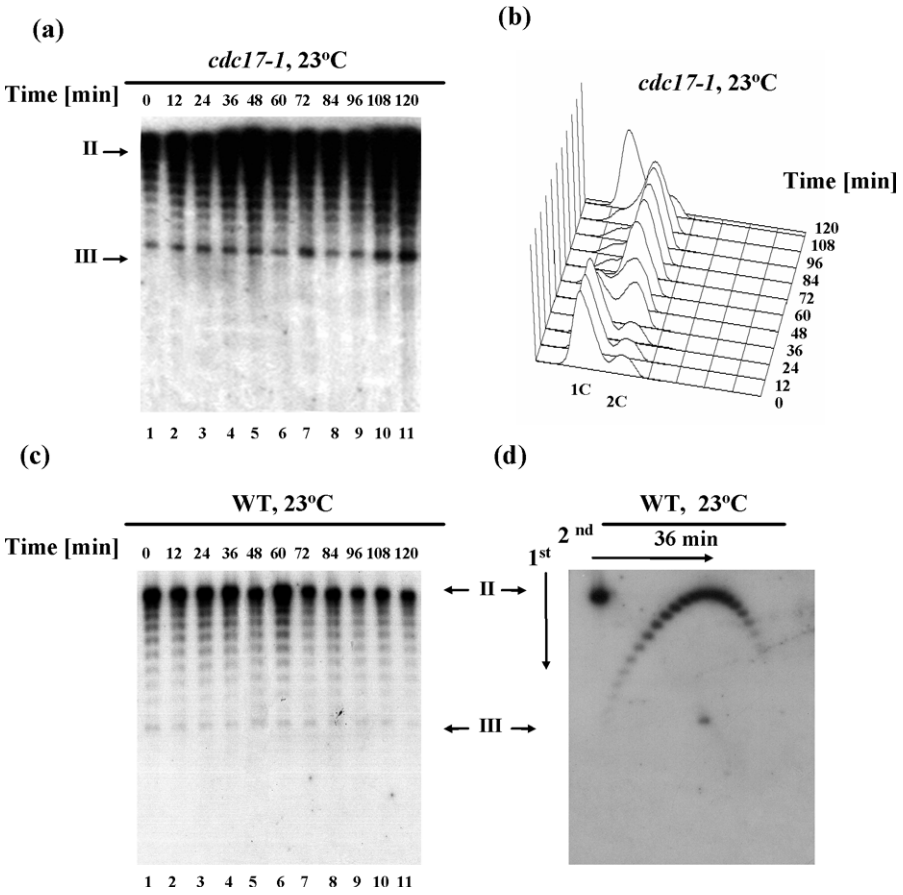

Figure 1. Topoisomer distribution of the 2 μm plasmid during the cell-cycle progression. (a) Chloroquine/agarose gel electrophoresis of DNA samples from 2765-1-4a (*cdc17-1*) strain, taken at 12 minute intervals after α pheromone release (the 0 minute sample is taken immediately after the release from the blocking agent). (b) FACS analysis of DNA contents of the 2765-1-4a (*cdc17-1*) strain during the cell-cycle progression. (c) Chloroquine/agarose gel electrophoresis of DNA samples from 7859-10-2a (WT) strain, taken at the indicated intervals after α pheromone release. (d) Two-dimensional chloroquine/agarose gel electrophoresis of DNA sample from 7859-10-2a (WT) strain, taken at the 36th minute after α pheromone release at 23 °C. DNA is transferred to nylon membrane and hybridized with specific random primed 2 μm DNA probe. Nicked DNA (II) and linear DNA (III) are pointed with arrows.

additional supercoiling during replication of DNA, because the whole DNA synthesis of the 2 μm molecule continues for two minutes only and the duration of origin unwinding is much shorter.

The 2 μm plasmid gains additional negative supercoiling in cells deficient in polymerase α activity

To overcome the limited time of origin unwinding and to measure the topological changes of DNA at that time, we used *S. cerevisiae cdc17-1* mutant cells. The *cdc17-1* temperature-sensitive mutation, encoding the catalytic subunit of DNA polymerase α, is known to diminish strongly the synthesis of DNA and leads to cell-cycle arrest at the beginning of the S phase.[32] The primase is not bound to the chromatin in the arrested cells.[14] RPA is attached to the origins of replication and when the arrest is prolonged, it also binds to neighbouring sequences.

Yeast cells bearing the *cdc17-1* mutation were synchronized with α pheromone and then shifted to restrictive temperature for one hour to turn the polymerase α activity off. After release from the α pheromone, still at the restrictive temperature, cells were arrested with single DNA content (1C) (Figure 2(c)). DNA was isolated and gel electrophoresis in the presence of chloroquine was performed. Apart from the normal distribution of topoisomers of the 2 μm plasmid, the presence of a fast-migrating form was observed (Figure 2(a), lanes 3 and 4).

A more detailed analysis of the topology of the plasmid from the *cdc17-1* strain shifted to restrictive temperature was carried out by two-dimensional agarose gel electrophoresis in the presence of chloroquine (Figure 2(b)). Figure 2(b) shows that the fast-migrating form of interest has taken place as the last one counterclockwise around the resulting arc, which shows that this form is the most negatively supercoiled. By analogy with previous data from *in vitro* experiments,[24,25] we refer to it as a U form of DNA. Furthermore, the Figure shows that there is a significant amount of DNA to the left of this form, consisting of highly negatively supercoiled species.

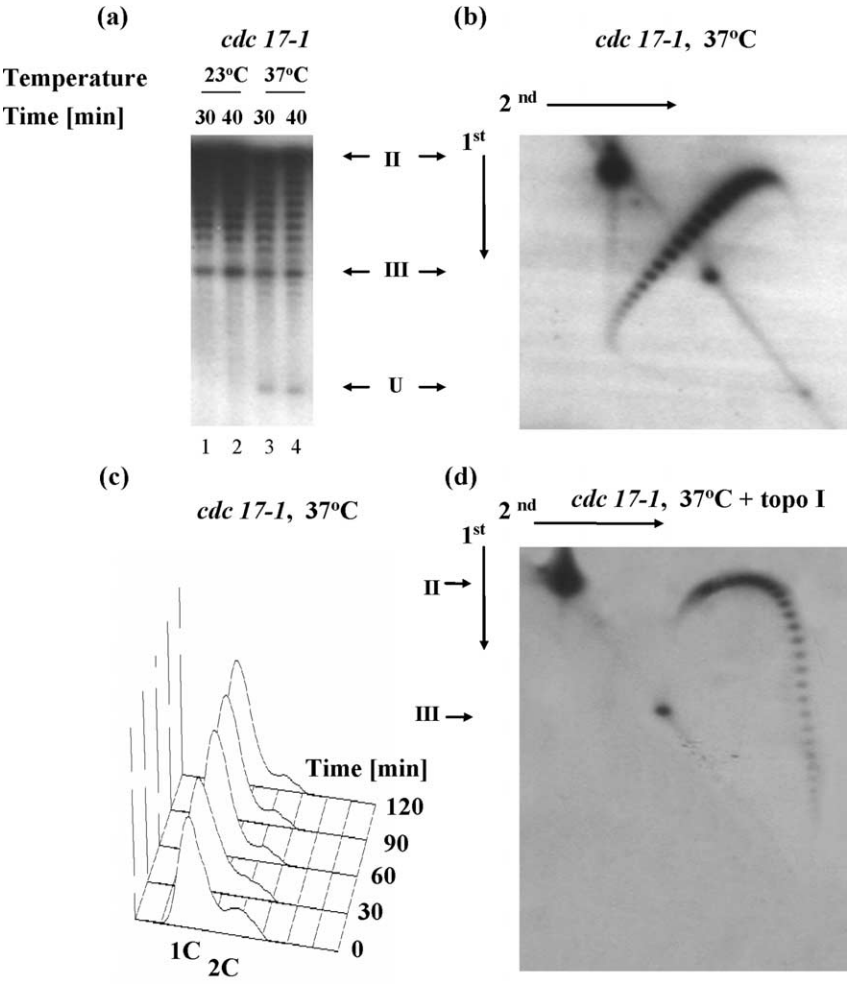

Figure 2. Topological pattern of the 2 μm minichromosome in *S. cerevisiae* cells deficient in polymerase α activity. (a) Chloroquine/agarose one-dimensional gel electrophoresis of DNA samples from strain 2765-1-4a (*cdc17-1*), taken at 30 minutes (lane 3) and 40 minutes (lane 4), after α pheromone release at 37 °C (0 minute). Lanes 1 and 2 show the topoisomer distribution of the plasmid at 30 minutes and 40 minutes, not shifted to 37 °C. (b) DNA sample (a, lane 3), subjected to two-dimensional chloroquine/agarose gel electrophoresis. The positions of the nicked DNA (II), linear DNA (III) and U form DNA of the 2 μm plasmid are shown. (c) FACS analysis of strain 2765-1-4a, synchronized in G1 and shifted to 37 °C. (d) DNA sample (a, lane 3), treated with calf thymus topoisomerase I and run on a two-dimensional chloroquine/agarose gel.

To confirm that the last band counterclockwise around the arc of topoisomers (Figure 2(b)) is a highly negatively supercoiled form, we used calf thymus topoisomerase I. This enzyme relaxes both positive and negative supercoiling.[34] The *cdc17-1* DNA sample, shifted to restrictive temperature, as described above, was treated with topoisomerase I and analysed by two-dimensional chloroquine/agarose gel electrophoresis (Figure 2(d)). The resulting topoisomeric pattern showed the disappearance of the U form DNA, as well as of the other highly negatively supercoiled species from the left part of the arc. New topoisomeric forms appeared to the right of the arc. They corresponded to relaxed forms, and weakly negatively and positively supercoiled forms of the plasmid. This confirms the suggestion that the observed U form is highly negatively supercoiled DNA.

Generation of U form DNA does not require primer synthesis

To ensure that the U form DNA is not a result of RNA primer synthesis, we examined the rate of supercoiling of the 2 μm minichromosome in a yeast strain deficient in primase activity. Temperature-sensitive mutations in the 68 kDa subunit (*pri2*) lead to lack of polymerase α association with chromatin and RNA primer synthesis defects during the initiation of DNA replication.[2]

Pri2-1 mutant *S. cerevisiae* cells[35] were blocked at the M phase of the cell-cycle by treatment with nocodazole for 2.5 hours at 23 °C. Then the culture was transferred to a restrictive temperature for 30 minutes. The nocodazole was removed from the medium and the incubation continued at 37 °C. Samples were taken at different time-points. Total DNA was isolated from the samples and one-dimensional chloroquine/agarose gel electrophoresis was carried out. The analysis of the topoisomer distribution of the 2 μm DNA plasmid (Figure 3(a)) revealed the appearance of the U form DNA one hour after the removal of the blocking agent, which corresponds to the end of the G1 phase and the beginning of the S phase. This result indicates that the generation of U form DNA does not require primer synthesis.

Additional negative supercoiling of a centromeric plasmid in cells deficient in primase activity

In the experiments described above, the 2 μm DNA plasmid was examined. The 2 μm

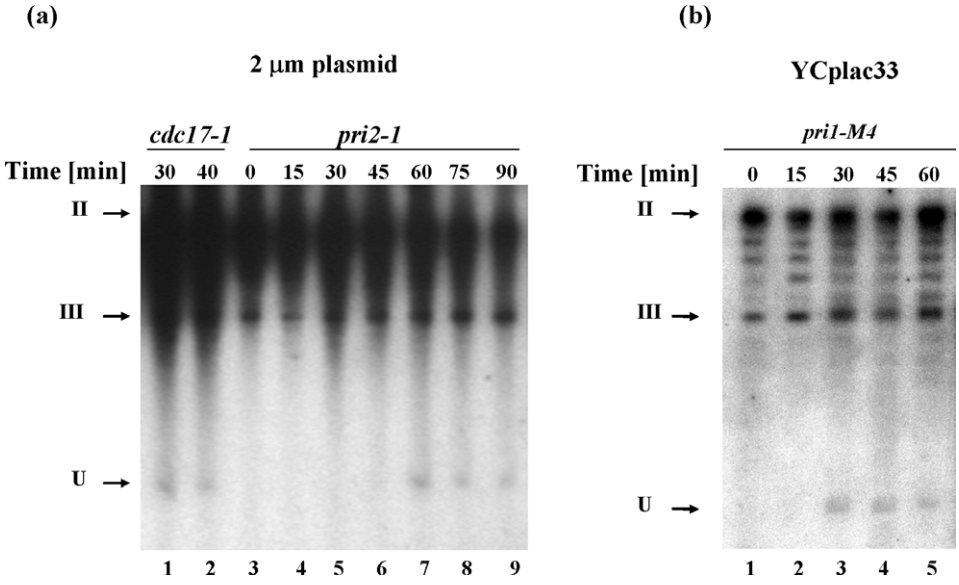

Figure 3. Topological pattern of DNA plasmids in *pri 2-1* and *pri1-m4* mutant strains. (a) Lanes 1 and 2 show the topology of the 2 μm plasmid at 30 minutes and 40 minutes, respectively, after α pheromone release at 37 °C of the 2765-1-4a (*cdc17-1*) mutant cells. Lanes 3–9 represent the topological pattern of the 2 μm minichromosome in *pri 2-1* deficient *S. cerevisiae* YB 421 cells at 0, 15 minutes, 30 minutes, 45 minutes, 60 minutes, 75 minutes and 90 minutes after the removal of the nocodazole at 37 °C. The positions of the nicked DNA (II), linear DNA (III) and U form DNA of the 2 μm plasmid are shown. (b) Lanes 1–5 present the topological pattern of DNA samples taken at 0 minutes, 15 minutes, 30 minutes, 45 minutes, and 60 minutes after α pheromone release at 37 °C of the YCplac33 from the transformed Cy 387 (*pri1-m4*) strain. The positions of the nicked DNA (II), linear DNA (III) and U form DNA of the YCplac33 plasmid are indicated with arrows.

minichromosome does not contain a centromere sequence, it has a mechanism for copy-number amplification based on double-rolling-circle replication after site-specific recombinational reversion of the replication fork following a single initiation event.[36]

This complicated mechanism raises the possibility that U form DNA may be idiosyncratic to the 2 μm plasmid. To address this possibility, we carried out the same kind of experiment for the YCplac33 plasmid, transformed into *pri2-1* and *pri1-m4* mutant yeast cells. This plasmid contains the CEN4 centromere sequence and the ARS1 origin of replication.[37] Its replication occurs just once per cell-cycle during S phase.

The *pri1-m4* is a temperature-sensitive mutation of the 48 kDa subunit of the yeast primase, deficient in the early step of DNA synthesis.[35] After chloroquine/agarose gel electrophoresis, U form DNA was observed in both *pri1-m4* (Figure 3(b))

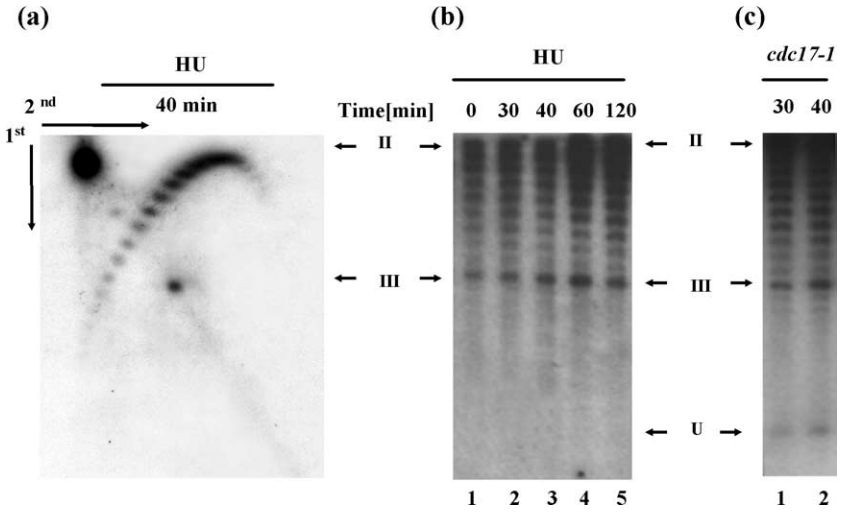

Figure 4. Hydroxyurea-treated *S. cerevisiae* cells do not show the U form of the 2 μm minichromosome. (a) Two-dimensional chloroquine/agarose gel electrophoresis of DNA sample from 2765-1-4a (*cdc17-1*) strain, taken at the 40th minute after α pheromone release at 23 °C in the presence of hydroxyurea (HU). (b) One-dimensional gel electrophoresis of DNA samples from *cdc17-1* strain, taken at the indicated times after α pheromone release at 23 °C in the presence of hydroxyurea. (c) Chloroquine/agarose gel electrophoresis of DNA samples from 2765-1-4a (*cdc17-1*) cells, taken after α pheromone release at 37 °C. Hybridisations are made with 2 μm plasmid specific probe. The positions of the nicked DNA (II), linear DNA (III) and U form DNA are indicated with arrows.

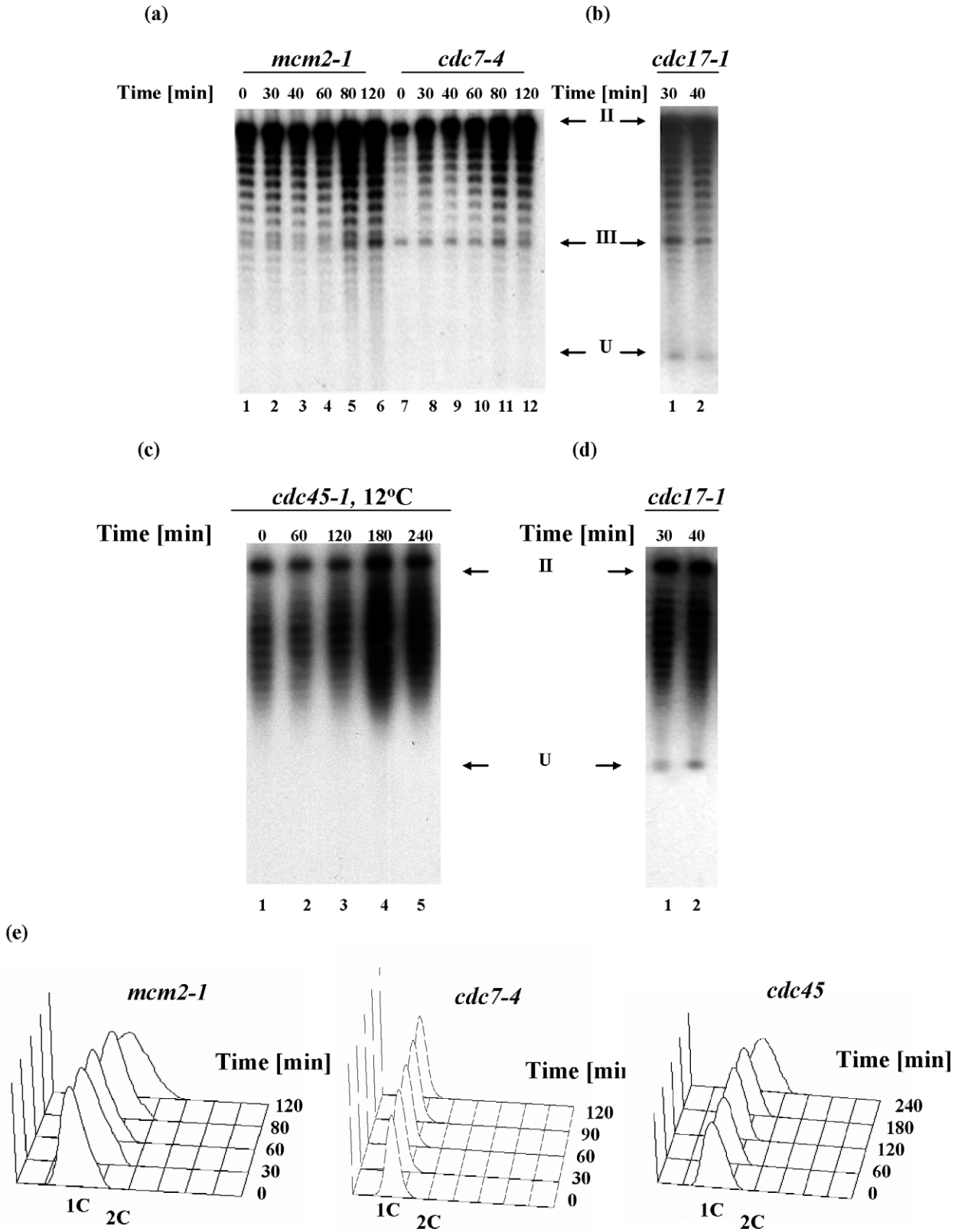

Figure 5. No U form is generated of the 2 μm plasmid in mutant *mcm2-1*, *cdc45-1* and *cdc7-4* strains. (a) Chloroquine/agarose gel electrophoresis of DNA samples from YB 560 (*mcm2-1*) and YB 291 (*cdc7-4*) strains, taken at the indicated time-points after the α pheromone release at 37 °C (0 minute). (b) Topological pattern of DNA samples from 2765-1-4a (*cdc17-1*) cells, taken at 30 minutes and 40 minutes after α pheromone release at 37 °C. (c) Chloroquine/agarose gel electrophoresis of DNA samples from YB 298 (*cdc45-1*) strain, taken at the indicated time-points after α pheromone release at 12 °C. (d) Topological pattern of DNA samples from 2765-1-4a (*cdc17-1*) cells, taken at 30 minutes and 40 minutes after α pheromone release at 37 °C. The concentration of chloroquine in the gel electrophoresis (c) and (d) is 4.0 μg/ml. Hybridisations are made with a 2 μm plasmid-specific probe. The positions of the nicked DNA (II), linear DNA (III) and U form DNA of the plasmid are indicated with arrows. (e) FACS analysis of strains YB 560 (*mcm2-1*), YB 291 (*cdc7-4*) and YB 298 (*cdc45-1*), synchronized in G1 and shifted to restrictive temperature.

and *pri2-1* (data not shown). These data demonstrate that the U form DNA is not limited to the 2 μm plasmid.

The observed U form DNA is not a partially replicated plasmid intermediate

To rule out the possibility that the U form DNA is a result of residual DNA synthesis, we analysed the topological changes of the 2 μm plasmid in yeast cells, treated with hydroxyurea (HU), which inhibits ribonucleotide reductase and thus reduces the concentration of deoxyribonucleotides in the cell. The early origins are activated, but the replication fork stalls after synthesis of DNA fragments of various lengths.[22] Wild-type cells, synchronized with α pheromone, were incubated with HU for an hour. After washing out the α pheromone, the cells were released into a medium containing HU. DNA was isolated from samples taken at certain intervals and one-dimensional chloroquine/agarose gel electrophoresis was performed (Figure 4(b)). The 40th minute probe was run on a two-dimensional chloroquine/agarose electrophoresis (Figure 4(a)). The U form of the 2 μm minichromosome was not detected. These results indicate that the observed U form DNA is not a partially replicated plasmid intermediate.

No U form DNA is generated in *mcm2-1*, *cdc45-1* or *cdc7-4* mutant strains

The MCM complex is required for the initiation of DNA replication. The MCM helicase activity suggests its key role in origin unwinding.[38,39] To enquire whether an active MCM complex is required for U form DNA generation, the *mcm2-1* mutant strain, deficient in MCM activity,[40] was synchronized with α pheromone and released at the restrictive temperature. The *mcm2-1* mutation is known to affect the interaction of Mcm2 and Dbf4 during the initiation process.[41] Our *mcm2-1* fluorescence-activated cell sorter (FACS) results (Figure 5(e)) as well as those of Yan and colleagues,[42,43] indicate a slow shift toward a condition between 1C and 2C of DNA but lack of completion of replication.

The rate of supercoiling was analyzed by chloroquine/agarose gel electrophoresis. U form DNA formation was not detected (Figure 5(a)).

Cdc45 associates with the origin at the late G1 phase of the cell-cycle and transforms the pre-RC into the pre-initiation complex (pre-IC).[6,11] It is required for the chromatin association of DNA polymerase α.[44,45] We decided to check whether the Cdc45 is required for the replicative DNA unwinding in *S. cerevisiae*. *cdc45-1* mutant yeast cells[46] were synchronized with α pheromone and released at the restrictive temperature (12 °C). The flow cytometry results for the *cdc45-1* strain (Figure 5(e)) show that three hours after the release from the blocking agent at the restrictive temperature, the cells are arrested with 1C of DNA. After that point, they start slowly to migrate towards 2C. Our FACS results, as well as those of Zou and co-workers,[47] indicate that until the third hour the mutated Cdc45 protein does not allow initiation of DNA replication. DNA was isolated from samples taken at different time-points and the topology of the 2 μm plasmid was examined. The results (Figure 5(c)) showed the lack of extremely negatively supercoiled DNA.

At late G1 phase, the activation of the Cdc7/Dbf4 protein kinase[11] is necessary to trigger the pre-IC to unwind DNA origin. We examined the yeast *cdc7-4* mutant strain, deficient in Cdc7/Dbf4 activity, for U form DNA formation. After release at the restrictive temperature, the *cdc7-4* mutant strain arrests with unreplicated DNA (Figure 5(e)). Figure 5(a) indicates that no U form of the 2 μm plasmid was observed after chloroquine/agarose gel electrophoresis (Figure 5(a)).

The lack of additional negative supercoiling of the 2 μm plasmid for *mcm2-1*, *cdc45-1* and *cdc7-4* mutant cells is consistent with the suggestion that U form DNA is not generated during the pre-IC formation until the activation of Cdc7 protein kinase.

Interaction of Tof1, Csm3 and Mrc1 checkpoint proteins with MCM helicase complex

The amount of U form DNA observed in this work was small. We therefore explored the possibility that the generation of this form is suppressed by the DNA replication checkpoint. It is known that RAD53-mediated checkpoint mechanisms slow DNA replication, induce inhibition of late origin firing and delay cell-cycle progression in response to either DNA damage or HU replication block.[20–22] Recent results suggest that single-stranded DNA is the initial signal that activates the checkpoint.[48,49]

The replicative unwinding observed in polymerase α/primase mutants will generate single-stranded DNA regions, which could cause checkpoint inhibition of still unreplicated origins, thereby decreasing U form DNA formation. If so, the amount and extent of unwinding in polymerase α/primase mutant in *mec1*- or *rad53*-deficient cells should be increased. However, we were not able to create either viable temperature-sensitive or inducible degron mutants of polymerase α/primase subunits in *mec1* or *rad53*-deficient yeast strains (Table 1). Notably, it has been shown that the temperature-sensitive double mutants *pri1-4*/*rad53* and *pri1-4*/*mec1* are not viable,[35] indicating hypersensitivity to mutation combining defects in DNA synthesis and replication checkpoint.

To approach these issues from a different angle, we looked for physical interactions between replication checkpoint proteins and the DNA unwinding machinery. Recently, it was shown that Mrc1 and Tof1, proteins, which are required for activation of replication checkpoint,[48,50–53] travel with the replication fork.[54–56] We therefore studied the possible interactions between the replication checkpoint complexes and the DNA helicase. Protein

Table 1. Construction of double mutants of various polymerase α/primase and S-phase checkpoint genes by combining inducible degrons (td), temperature-sensitive (ts) and deletion mutants

	Δ *mec1*/Δ *sml1*	Δ *rad53*/Δ *sml1*	Δ *tof1*	Δ *csm3*	Δ *mrc1*
Ts *cdc17-1*	Inviable	Inviable	Inviable	Inviable	Inviable
td *cdc17*	Inviable	Inviable	Inviable	Inviable	Inviable
td *pri 1*	Inviable	Inviable	Inviable	Inviable	Inviable
td *pri 2*	Inviable	Inviable	Inviable	Inviable	Inviable

interactions were documented by C-terminal tandem affinity purification (TAP)-tags on Tof1, Mrc1, Rad53 and N-terminal TAP-tags on Mec1 and Tel1, because strains containing C-terminal TAP-tagged Mec1 or Tel1 were not viable, followed by two-step affinity chromatography (Figure 6).[57] In this experiment, all visible bands were identified by mass spectrometry. Only specific bands are labeled in the Figures. The other bands are a subset of proteins that were detected repeatedly using the TAP tag and hence are common background contaminants.[58]

Mass spectrometry identifications show that Tof1 and Csm3 co-purify together in stoichiometric amounts (Figure 6(a) and (b)), consistent with recent findings of their interaction in *S. cerevisiae*[59] and *Schizosaccharomyces pombe*.[60] Tof1 and Csm3 were also found to interact with Mrc1 in substoichiometric amounts (Figure 6(a)–(c)). In addition, the three checkpoint proteins interact with subunits of the MCM helicase complex (Figure 6(a)–(c)). Csm3 was co-purified with Mcm7, Tof1 was co-purified with Mcm6, and Mrc1 was co-purified with Mcm2 and Mcm3. The fact that the three checkpoint proteins co-purify with different MCM subunits may indicate specific interaction with these subunits; however, it may also reflect certain limitation of sensitivity. We were not able to detect direct interaction between Tof1, Csm1 or Mrc1 with Cdc45, protein, which interacts with the MCM

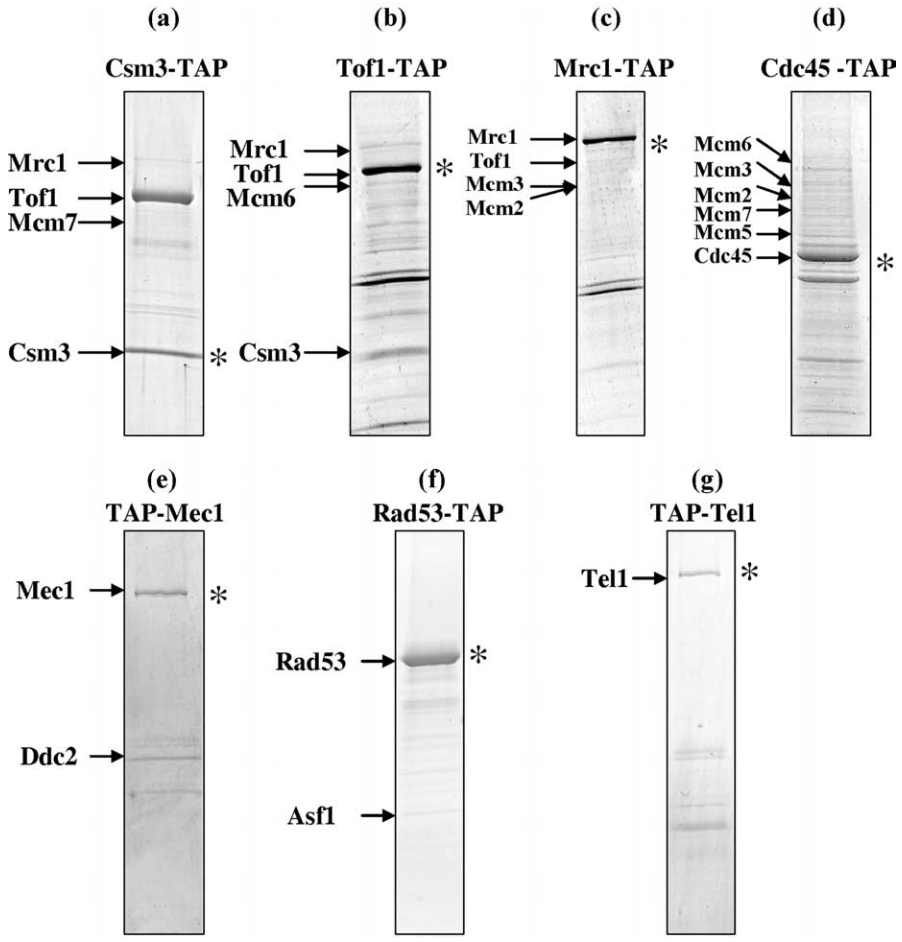

Figure 6. Checkpoint protein complexes. Coomassie brilliant blue-stained SDS/polyacrylamide-gradient gel electrophoresis of (a) Tof1, (b) Mrc1, (c) Csm3, (d) Cdc45 1, (e) Mec1, (f) Rad53, and (g) Tel1 protein complexes. TAP-tagged proteins are indicated with an asterisk (*). Bands corresponding to background contaminants common to the TAP methodology[61] are not labelled.

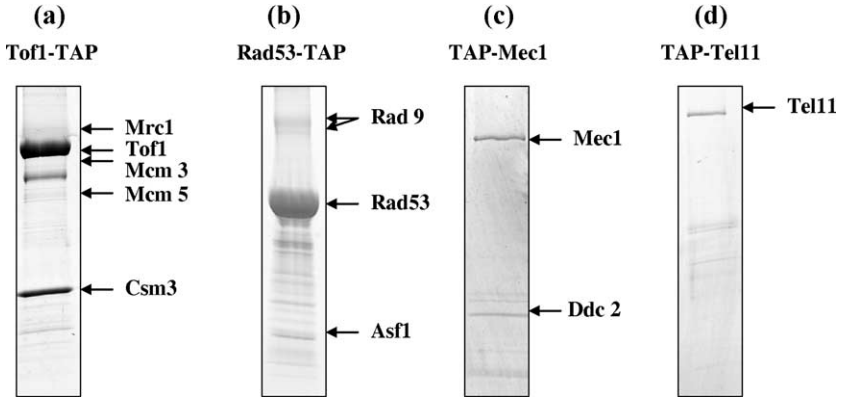

Figure 7. Checkpoint protein complexes during hydroxyurea replication and DNA damage block. Coomassie brilliant blue-stained SDS/polyacrylamide-gradient gel electrophoresis of (a) Tof1, (c) Mec1, and (d) Tel1 protein complexes during hydroxyurea replication block. (b) Rad53 protein complexes during MMS replication block. TAP-tagged proteins are indicated with an asterisk (*). Bands corresponding to background contaminants common to the TAP methodology[58] are not labelled.

complex. To validate this result, the Cdc45 TAP–tag protein complex was purified (Figure 6(d)). The interaction between Cdc45 and the MCM helicase complex was confirmed but no co-purification with Tof1 or Mrc1 was observed. Interaction of Rad53 with Asf1 and of Mec1 with Ddc2 were confirmed,[61–63] but no co-purification with helicase complex was observed. No interaction with Tel1 was found (Figure 6(g)).

In order to identify possible rearrangements of checkpoint complexes during stalling of replication fork, the Tof1, Mec1 and Tel1 complexes were purified during HU replication block[22] and Rad53 was purified after treatment of cells with methylmethane sulphonate (MMS). As seen in Figure 7(a), during HU block, Tof1 interacts with Csm3, Mrc1, and Mcm3 and Mcm5 subunits of the MCM complex. The co-purification of Tof1 with Mcm3 and Mcm5 during the HU replication block but not with Mcm6 (which is presented in Tof1 complex during normal replication) either presents regulatory change or variability of identifications at these lower limits of sensitivity. As expected, MMS promotes the interaction of Rad9 with Rad53 (Figure 7(b)),[61,62] indicating that the experimental conditions were satisfactory. No other change between interactions of checkpoint proteins during normal replication and replication block was observed (Figure 7(c) and (d)).

These results suggest that Tof1/Csm3/Mrc1 complex interacts directly with MCM helicase but not with cdc45 during both replication fork progression and stalled DNA replication. The stoichiometric interaction of Tof1 with Csm3 indicates that this complex is present during replication and throughout the cell-cycle. In contrast, the non-stoichiometric amounts of MCM-complex and Mrc1 found with Tof1 and Csm3 preparations suggest that these interactions are present only during DNA replication. In order to investigate the genetic interaction between *tof1*, *csm3*, *mrc1* and *cdc17-1* mutants, in which DNA unwinding becomes uncoupled from DNA synthesis, we tried to create double *cdc17-1*/Δ*tof1*, *cdc17-1*/Δ*csm3* and *cdc17-1*/Δ*mrc1* mutant strains. None was viable (Table 1), which suggests that the Mrc1/Tof1/Csm3 checkpoint complex can prevent lethality of cells in which DNA unwinding can proceed without DNA synthesis.

Discussion

The extensive negative supercoiling is a result of replicative DNA unwinding

Here, we demonstrate that some plasmid molecules gain additional negative supercoiling in mutant polymerase α/primase cells, deficient in DNA synthesis. Such supercoiling was detected in yeast strains exhibiting disorders in the 180 kDa polymerase α as well as in the 48 kDa and 68 kDa primase subunits. In all mutant strains, additional negative supercoiling appeared during the G1/S phase transition. At that time, in cells with normal polymerase α/primase activity, such extra supercoiling was not observed. No change in the topology of plasmid DNA was observed in *mcm2-1*, *cdc45-1*, *cdc7-4* or HU-arrested yeast cells. As the replication intermediates take place closer to the gel starts when subjected to chloroquine/agarose gel electrophoresis (demonstrated by *in vitro* experiments),[25] our results indicate that the extensive negative supercoiling of DNA is a result of replicative unwinding, which is not followed by DNA synthesis *in vivo*. Such supercoiling was observed in both the 2 μm minichromosome and a plasmid in which the replication is initiated at the chromosomal origin ARS1. This indicates that the extra supercoiling is representative of the chromosomal origin of DNA unwinding.

The replicative unwinding of DNA in cells deficient in polymerase α activity is at least 400 bp

To determine the length of DNA origin unwinding, we took into account the fact that the difference in supercoiling of two neighbouring topoisomers from the arc of distribution is one supercoil. One negative supercoil is a result of 10 bp of unwound DNA. Figure 2(b) makes it obvious that the distance

between two neighbouring topoisomers in the lower left part of the arc decreases as their negative supercoiling is increased and they fuse in an uninterrupted line. For this reason, it is difficult to count bands in that region of the arc. Considering the distances between the final separable negative topoisomers in Figure 2(b), we suggest that the distance between the last common negative topoisomers for the shifted and not shifted to 37 °C *cdc17-1* plasmid DNA and the U form is at least 40 bands (compare Figure 2(a), line 1 and (b)). Therefore, the 2 μm plasmid gains at least 40 additional negative supercoils, corresponding to a minimum of 400 bp of unwound origin DNA.

DNA unwinding and polymerase α/primase complex

It is known that leading strand DNA synthesis is initiated at a discrete starting point.[17] The extensive unwinding of DNA, shown here, is too large to place the polymerase α/primase complex at a specific location at the origin of replication. Besides DNA unwinding, interaction of polymerase α/primase with ORC is likely required for the exact positioning and initiation of DNA replication.[53] The synthesized RNA/DNA primer at the replication origin is elongated by the processive polymerases δ and ε. This requires the continuance of the DNA unwinding throughout the elongation of replication. The leading strand DNA synthesis and DNA unwinding are normally coupled. This poses the question of whether the leading strand synthesis is required for DNA unwinding. If that were true, the unwinding in the lack of DNA synthesis would be limited to a region around the replicative starting site. The extensive unwinding detected in this work demonstrates that *in vivo* unwinding of DNA during elongation does not require DNA synthesis. Our results indicate that the DNA unwinding also does not require polymerase α/primase complex, because it has been shown that in the *pri2-1* and *pri1-m4* mutant strains, polymerase α/primase complex was not loaded onto the chromatin[2]. Nevertheless, the plasmid molecules from these strains showed extensive negative supercoiling as a result of DNA unwinding.

The limited number of unwound plasmid molecules and the direct interaction between the Tof1/Csm3/Mrc1 checkpoint proteins and the MCM helicase complex suggest a new mechanism for activation of the replication checkpoint.

During a replication block, yeast cells engage the Mec1/Rad53 checkpoint response, which prevents the collapse of stalled replication forks and allows the restart of DNA replication after recovery. This response requires both Tof1 and Mrc1 proteins, which were found to travel with the replication fork,[54,55] and phosphorylation of Mrc1 by Mec1. Uncoupling of the replicative unwinding from DNA synthesis and the direct interaction of Tof1/Csm3 and Mrc1 checkpoint proteins with the MCM helicase complex suggests a possible mechanism for activation of the replication checkpoint. Pausing of replicative DNA synthesis in response to decreasing levels of nucleotides or DNA damage leads to accumulation of single-stranded DNA regions as a result of the uncoupling of DNA unwinding from synthesis. RPA mediates Mec1/Ddc1 binding to the single-stranded regions[56] and allows Mec1 to phosphorylate Mrc1 to stabilize the MCM complex and stop the progression of DNA unwinding. Mec1-dependent activation of Rad53 leads to additional phosphorylation of Mrc1 and inhibition of still unreplicated origins. Keeping the MCM complex intact allows for restart of the replication fork when the replication block is relieved. Several other lines of evidence support this mechanism.

First, DNA damage by treatment with MMS reduces the rate of DNA replication fork progression.[65] Mec1 and Rad53 mutants show the same phenotype but initiate new DNA synthesis randomly in the regions ahead of the replication fork that do not contain active replication origins. In this case, slowing synthesis of the leading strand DNA would create single-stranded DNA by uncoupling helicase action and DNA synthesis, thus producing a template for random initiation of DNA replication. Second, during collapse of replication forks in Tof1 and Mrc1 mutants, Cdc45 and the MCM complex become uncoupled from DNA synthesis.[54] Third, depletion of the Mec1 homologue ATR increases plasmid DNA unwinding in *Xenopus* egg extracts.[55] Fourth, MCM is the only replication protein complex that, once unloaded, cannot reload onto the replication fork during S phase and restore DNA replication.[19]

Materials and Methods

Strains and media

Six *S. cerevisiae* strains were used in this work: 2765-1-4a (*MATa*, *cdc 17-1*, *his 7*, *leu2-3*, *112*), A364 background; 7859-10-2a (*MATa*, *his 7*, *leu2-3*, 112, *trp1-289*, *ura3-52*), wild-type, A364 background; YB 421 (*MATα*, *pri2−1* *leu2-3*, *112*, *trp 1-1*, *ura3-1*, *his3-11*, *15*, *can1-100*, *ade2-1*), W303-1B background; YB 560 (*MATa*, *mcm2-1*, *leu2-3*, *112*, *trp 1-1*, *ura3-1*, *his3-11*, *15*, *can1-100*, *ade2-1*), W303 background; YB 298 (*MATa*, *cdc45-1*, *leu2-3*, *112*, *trp 1-1*, *ura3-1*, *his3-11*, *15*, *can1-100*, *ade2-1*), W303-1A background; YB 291 (*MATa*, *cdc7-4*, *leu2-3*, *112*, *trp1-1*, *ura3-1*, *his3-11*, *15*, *ade2-1*), W303 background and isogenic to K699 Cy 387(*MATa*, *ade2-1*, *trp1-1*, *can1-100*, *leu2-3*,*112*, *his3-11*,*15*, *ura3*, *pri1-m4*), transformed with YCplac33. Cells from 2765-1-4a, YB 421, YB 560, YB 298 and YB 291 were grown in YPD medium (1% (w/v) yeast extract (Difco), 2% (w/v) Bacto-peptone (Difco), 2% (w/v) dextrose). The strain transformed with YCplac33 Cy 387 was grown in a minimal medium, containing 0.67% (w/v) Bacto-yeast nitrogen base, 2% dextrose, 20 mg/l of adenine sulphate, 20 mg/l of tryptophan, 30 mg/l of leucine and 20 mg/l of histidine–HCl for *ura3* selection.

Cell-cycle experiments

For α factor block and release experiments, cells were

suspended in liquid YPD medium to a density of approximately 1×10^7 cells/ml and α pheromone (Sigma) was added to a concentration of 10 μg/ml. Cells were arrested for two hours at 23 °C, washed free from the pheromone and released at 23 °C into fresh YPD medium containing 0.1 mg/ml of Pronase E (Sigma). The 0 minute time-point was taken immediately after the removal of the α pheromone. Samples were taken at intervals of 12 minutes.

The *cdc17-1* (2765-1-4a), *pri1-m4* (Cy 387), *mcm2-1* (YB 560) and *cdc7-4* (YB 291) temperature-sensitive mutants were first arrested with α factor at 23 °C (as described above) and then transferred to medium at 37 °C and kept in α factor for one hour. The α pheromone was removed by changing the medium, and 0.1 mg/ml of Pronase E was added. The incubation continued at 37 °C.

The *cdc45-1* (YB 298) temperature-sensitive mutant was first arrested with α factor at 23 °C and then transferred to 12 °C and kept in the presence of α factor for one hour. The α pheromone was removed by changing the medium, and 0.1 mg/ml of Pronase E was added. The incubation continued at 12 °C

For the *pri2-1* (YB 421) block experiments, cells were suspended in liquid YPD medium to a density of approximately 1×10^7 cells/ml and 10 μg/ml of nocodazole (Sigma) was added. Cells were arrested for 2.5 hours at 23 °C. Then the culture was transferred to the restrictive temperature (37 °C) for 30 minutes. The nocodazole was removed from the medium and the incubation continued at 37 °C.

For the HU arrest, cells were first synchronized with α pheromone at 23 °C, as described above and next transferred to a medium containing 0.2 M HU as well as α factor. After one hour the pheromone was washed away and the cells were resuspended in fresh YPD medium with HU and incubated at 23 °C.

FACS analysis was performed as described.[4]

DNA preparation and chloroquine/agarose gel electrophoresis

The preparation of *S. cerevisiae* DNA was as described,[56] and the samples were treated with RNase A and Proteinase K.[57] The plasmid relaxation by calf thymus topoisomerase I was carried out according to the Amersham Pharmacia protocol.

Electrophoresis was carried out as described.[30,31] For two-dimensional gel electrophoresis, DNA samples were applied to a 20 cm×20 cm×0.5 cm horizontal slab 0.7% (w/v) agarose gel. Electrophoresis in the first dimension was carried out at room temperature in circulating TBE (50 mM Tris–borate (pH 8.3), 1 mM EDTA) containing 0.8 μg/ml of chloroquine diphosphate. After 20 hours at 1.5 V cm^{-1}, the gel was soaked for four hours in TBE containing 4.0 μg/ml of chloroquine diphosphate. After 90° rotation of the slab, electrophoresis in the second dimension was carried out at room temperature for 14 hours at 1.5 V in fresh buffer. Chloroquine/agarose one-dimensional gel electrophoresis was carried out in the same manner as the first dimension of the two-dimensional electrophoresis unless noted otherwise.

Processing and transfer to nylon membrane were performed by standard protocols.[64] The hybridisation to random primed 2 μm minichromosome 1203 HindIII restriction fragment and YCplac 382bp BsaHI restriction fragment probes was carried out as described.[27]

Purification of protein complexes

Genes of interest were tagged by in-frame fusion of the open reading frames (ORFs) with a PCR-generated targeting cassette encoding for the TAP tag and a selectable marker.[57] Correct cassette integrations were confirmed by PCR and Western blot analysis. TAP purification was performed as described.[48,58] The purified protein assemblies were concentrated, separated by denaturing gradient gel electrophoresis, and visualized by staining with Coomassie brilliant blue.

Identification of proteins by mass spectrometry

Protein bands were excised and in-gel digested with trypsin (Promega, Madison WI) as described.[59] Aliquots of 1 μl were withdrawn from the digests and analyzed by peptide mass fingerprinting on a REFLEX IV mass spectrometer (Bruker Daltonics, Billerica, MA) on AnchorChip™ targets as described.[60] If no conclusive identification was achieved, peptides were extracted from the polyacrylamide gel matrix by 5% (v/v) formic acid and acetonitrile. The combined extracts were dried, and analyzed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer QSTAR Pulsar *i* (MDS Sciex, Concord, Canada) or by nanoflow liquid chromatography–tandem mass spectrometry on an ion trap mass spectrometer LTQ (Thermo-Electron corp., San Jose, CA). Peptide mass fingerprints and tandem mass spectra were searched against the MSDB database using Mascot software (Matrix Science Ltd, London) installed on a local server. All hits were statistically significant, according to their corresponding MOWSE scores, but were accepted upon manual inspection of corresponding matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) or tandem mass spectra.

Acknowledgements

The authors thank Joel Huberman, Marco Foiani and Bruce Stillman, who kindly provided the yeast strains. We thank Miroslav Velev and Lubomira Chakalova for their assistance in the preparation of the manuscript, and Pencho Venkov for his help and support. The research of S.S.S. in Germany was supported by a fellowship from the Alexander von Humboldt Foundation. This work was supported, in part, by National Council "Scientific Research" grant K-1306/03 and UNESCO grant UVO-POSTE 875.749.1.

References

1. Foiani, M., Lucchini, G. & Plevani, P. (1997). The DNA polymerase alpha–primase complex couples DNA replication, cell-cycle progression and DNA-damage response. *Trends Biochem. Sci.* **22**, 424–427.
2. Desdouets, C., Santocanale, C., Drury, L. S., Perkins, G., Foiani, M., Plevani, P. & Diffley, J. F. (1998). Evidence for a Cdc6p-independent mitotic resetting event involving DNA polymerase alpha. *EMBO J.* **17**, 4139–4146.

3. Diffley, J. F. (2004). Regulation of early events in chromosome replication. *Curr. Biol.* **14**, R778–R786.
4. Bell, S. P., Kobayashi, R. & Stillman, B. (1993). Yeast origin recognition complex functions in transcription silencing and DNA replication. *Science*, **262**, 1844–1849.
5. Santocanale, C. & Diffley, J. F. (1996). ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 6671–6679.
6. Aparicio, O. M., Weinstein, D. M. & Bell, S. P. (1997). Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell*, **91**, 59–69.
7. Tanaka, T., Knapp, D. & Nasmyth, K. (1997). Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell*, **90**, 649–660.
8. Tanaka, S. & Diffley, J. F. (2002). Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. *Nature Cell Biol.* **4**, 198–207.
9. Nishitani, H., Lygerou, Z., Nishimoto, T. & Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature*, **404**, 625–628.
10. Stillman, B. (1996). Cell-cycle control of DNA replication. *Science*, **274**, 1659–1664.
11. Zou, L. & Stillman, B. (1998). Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science*, **280**, 593–596.
12. Kamimura, Y., Tak, Y. S., Sugino, A. & Araki, H. (2001). Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *EMBO J.* **8**, 2097–2107.
13. Kelly, T. J. & Brown, G. W. (2000). Regulation of chromosome replication. *Annu. Rev. Biochem.* **69**, 829–880.
14. Tanaka, T. & Nasmyth, K. (1998). Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J.* **17**, 5182–5191.
15. Kamimura, Y., Masumoto, H., Sugino, A. & Araki, H. (1998). Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication. *Mol. Cell. Biol.* **18**, 6102–6109.
16. Masumoto, H., Sugino, A. & Araki, H. (2000). Dpb11 controls the association between DNA polymerases α and ε and the autonomously replicating sequence region of budding yeast. *Mol. Cell. Biol.* **20**, 2809–2817.
17. Bielinsky, A. K. & Gerbi, S. A. (1999). Chromosomal ARS1 has a single leading strand start site. *Mol. Cell.* **3**, 477–486.
18. Tercero, J. A., Labib, K. & Diffley, J. F. (2000). DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p. *EMBO J.* **19**, 2082–2093.
19. Labib, K., Tercero, J. A. & Diffley, J. F. (2000). Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science*, **288**, 1634–1647.
20. Weinert, T. A. & Hartwell, L. H. (1988). The RAD9 gene controls the cell-cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science*, **241**, 317–322.
21. Paulovich, A. G. & Hartwell, L. H. (1995). A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell*, **82**, 841–847.
22. Santocanale, C. & Diffley, J. F. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature*, **395**, 615–618.
23. Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M. *et al*. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. *Nature*, **41**, 557–561.
24. Dean, F. B., Bullock, P., Murakami, Y., Wobbe, C. R., Weissbach, L. & Hurwitz, J. (1987). Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. Natl Acad. Sci. USA*, **84**, 16–20.
25. Walter, J. & Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RP A, and DNA polymerase alpha. *Mol. Cell.* **5**, 617–627.
26. Geraghty, D. S., Ding, M., Heintz, N. H. & Pederson, D. S. (2000). Premature structural changes at replication origins in a yeast minichromosome maintenance (MCM) mutant. *J. Biol. Chem.* **275**, 18011–18021.
27. Huberman, J. A., Spotila, L. D. & Davis, L. R. (1987). The *in vivo* replication origin of the yeast 2 microns plasmid. *Cell*, **51**, 473–481.
28. Brewer, B. J. & Fangman, W. L. (1987). The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell*, **51**, 463–471.
29. Diffley, J. F. X., Cocker, J. H., Dowell, S. J. & Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins *in vivo*. *Cell*, **78**, 303–316.
30. Saavedra, R. A. & Huberman, J. A. (1986). Both DNA topoisomerases I and II relax 2 micron plasmid DNA in living yeast cells. *Cell*, **45**, 65–70.
31. Giaever, G. N. & Wang, J. C. (1988). Supercoiling of intracellular DNA can occur in eukaryotic cells. *Cell*, **55**, 849–856.
32. Budd, M. E., Wittrup, K. D., Bailey, J. E. & Campbell, J. L. (1989). DNA polymerase I is required for premeiotic DNA replication and sporulation but not for X-ray repair in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 365–376.
33. Shure, M., Pulleyblank, D. E. & Vinograd, J. (1977). The problems of eukaryotic and prokaryotic DNA packaging and *in vivo* conformation posed by superhelix density heterogeneity. *Nucl. Acids Res.* **4**, 1183–1205.
34. Ferro, A. M., Higgins, N. P. & Olivera, B. M. (1983). Poly(ADP-ribosylation) of a DNA topoisomerase. *J. Biol. Chem.* **258**, 6000–6003.
35. Marini, F., Pellicioli, A., Paciotti, V., Lucchini, G., Plevani, P., Stern, D. F. & Foiani, M. (1997). A role for DNA primase in coupling DNA replication to DNA damage response. *EMBO J.* **16**, 639–650.
36. Broach, J. R. & Volkert, F. (1991). Circular DNA plasmids of yeasts: genome dynamics, protein synthesis and energetics. In *The Molecular and Cellular Biology of the Yeast Saccharomyces*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY pp. 297–331.
37. Gietz, R. D. & Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.
38. You, Z., Komamura, Y. & Ishimi, Y. (1999). Biochemical analysis of the intrinsic Mcm4-Mcm6-Mcm7 DNA helicase activity. *Mol. Cell. Biol.* **19**, 8003–8015.
39. Lee, J. K. & Hurwitz, J. (2001). Processive DNA helicase activity of the minichromosome maintenance proteins 4, 6, and 7 complex requires forked DNA structures. *Proc. Natl Acad. Sci. USA*, **98**, 54–59.
40. Tye, B. K. (1999). MCM proteins in DNA replication. *Annu. Rev. Biochem.* **68**, 649–686.
41. Lei, M., Kawasaki, Y., Young, M. R., Kihara, M.,

Sugino, A. & Tye, B. K. (1997). Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev.* **11**, 3365–3374.
42. Yan, H., Gibson, S. & Tye, B. K. (1991). Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. *Genes Dev.* **5**, 944–957.
43. Yan, H., Merchant, A. M. & Tye, B. K. (1993). Cell-cycle-regulated nuclear localization of MCM2 and MCM3, which are required for the initiation of DNA synthesis at chromosomal replication origins in yeast. *Genes Dev.* **7**, 2149–2160.
44. Mimura, S. & Takisawa, H. (1998). Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. *EMBO J.* **17**, 5699–5707.
45. Aparacio, O. M., Stout, A. M. & Bell, S. P. (1999). Differential assembly of cdc45 and DNA polymerases at early and late origins of DNA replication. *Proc. Natl Acad. Sci. USA*, **96**, 9130–9135.
46. Hennessy, K., Lee, A., Chen, E. & Botstein, D. (1991). A group of interacting yeast DNA replication genes. *Genes Dev.* **5**, 958–969.
47. Zou, L., Mitchell, J. & Stillman, B. (1997). CDC45, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Mol. Cell. Biol.* **17**, 553–563.
48. Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bousset, K. *et al*. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nature Cell Biol.* **3**, 958–965.
49. Sogo, J. M., Lopes, M. & Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*, **297**, 599–602.
50. Foss, E. J. (2001). Tof1p regulates DNA damage responses during S phase in *Saccharomyces cerevisiae*. *Genetics*, **157**, 567–577.
51. Tanaka, K. & Russell, P. (2001). Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. *Nature Cell Biol.* **3**, 966–972.
52. Noguchi, E., Noguchi, C., Du, L. L. & Russell, P. (2003). Swi1 prevents replication fork collapse and controls checkpoint kinase Cds1. *Mol. Cell. Biol.* **23**, 7861–7874.
53. Vengrova, S. & Dalgaard, J. Z. (2004). RNase-sensitive DNA modification(s) initiates *S. pombe* mating-type switching. *Genes Dev.* **18**, 794–804.
54. Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T. *et al*. (2003). S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature*, **424**, 1078–1083.
55. Osborn, A. J. & Elledge, S. J. (2003). Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes Dev.* **17**, 1755–1767.
56. Zou, L. & Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA–ssDNA complexes. *Science*, **300**, 1542–1548.
57. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. & Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotechnol.* **17**, 1030–1032.
58. Shevchenko, A., Schaft, D., Roguev, A., Pijnappel, W. W. & Stewart, A. F. (2002). Deciphering protein complexes and protein interaction networks by tandem affinity purification and mass spectrometry: analytical perspective. *Mol. Cell. Proteomics*, **1**, 204–212.
59. Mayer, M. L., Pot, I., Chang, M., Xu, H., Aneliunas, V., Kwok, T. *et al*. (2004). Identification of protein complexes required for efficient sister chromatid cohesion. *Mol. Biol. Cell.* **15**, 1736–1745.
60. Noguchi, E., Noguchi, C., McDonald, W. H., Yates, J. R., 3rd & Russell, P. (2004). Swi1 and Swi3 are components of a fork protection complex in fission yeast. *Mol. Cell. Biol.* **24**, 8342–8355.
61. Sun, Z., Hsiao, J., Fay, D. S. & Stern, D. F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. *Science*, **281**, 272–274.
62. Vialard, J. E., Gilbert, C. S., Green, C. M. & Lowndes, N. F. (1998). The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. *EMBO J.* **17**, 5679–5688.
63. Rouse, J. & Jackson, S. P. (2000). LCD1: an essential gene involved in checkpoint control and regulation of the MEC1 signalling pathway in *Saccharomyces cerevisiae*. *EMBO J.* **19**, 5801–5812.
64. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1989). *Molecular Cloning: A Laboratory Manual* (2nd edit.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
65. Tercero, J. A. & Diffley, J. F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. *Nature*, **412**, 553–557.

Edited by J. Karn

(Received 16 September 2004; received in revised form 11 January 2005; accepted 13 January 2005)