The yeast Sgs1 helicase is differentially required for genomic and ribosomal DNA replication

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The members of the RecQ family of DNA helicases play conserved roles in the preservation of genome integrity. RecQ helicases are implicated in Bloom and Werner syndromes, which are associated with genomic instability and predisposition to cancers. The human BLM and WRN helicases are required for normal S phase progression. In contrast, *Saccharomyces cerevisiae* cells deleted for *SGS1* grow with wild-type kinetics. To investigate the role of Sgs1p in DNA replication, we have monitored S phase progression in *sgs1*Δ cells. Unexpectedly, we find that these cells progress faster through S phase than their wild-type counterparts. Using bromodeoxyuridine incorporation and DNA combing, we show that replication forks are moving more rapidly in the absence of the Sgs1 helicase. However, completion of DNA replication is strongly retarded at the rDNA array of *sgs1*Δ cells, presumably because of their inability to prevent recombination at stalled forks, which are very abundant at this locus. These data suggest that Sgs1p is not required for processive DNA synthesis but prevents genomic instability by coordinating replication and recombination events during S phase.
Keywords: DNA replication/genomic instability/rDNA/ RecQ helicases

Introduction

The *SGS1* gene of *Saccharomyces cerevisiae* encodes a DNA helicase of the RecQ family, which is involved in the maintenance of genome integrity in all organisms analyzed to date, from bacteria to human (Karow *et al*., 2000). Sgs1p is closely related to the human helicases BLM, WRN and RECQL4, defective in Bloom syndrome (BS), Werner syndrome (WS) and Rothmund–Thomson syndrome (RTS), respectively. These diseases are characterized by chromosome instability, hyper-recombination and an increased incidence of cancers (Mohaghegh and Hickson, 2001). However, the molecular events underlying these phenotypes have remained obscure.

New insights into the molecular basis of these disorders came with the finding of functional interactions between RecQ helicases and multiple components of the replication machinery, including proliferating cell nuclear antigen (PCNA), RP-A, topoisomerases and DNA polymerase δ (Gangloff *et al*., 1994; Watt *et al*., 1995; Lebel *et al*., 1999; Brosh *et al*., 2000; Wu *et al*., 2000). In yeast and human cells, levels of RecQ helicases peak in S phase (Dutertre *et al*., 2000; Frei and Gasser, 2000), and these enzymes were shown to co-localize with sites of ongoing DNA synthesis in yeast and in *Xenopus* (Frei and Gasser, 2000; Chen *et al*., 2001). Moreover, cells derived from BS and WS patients accumulate abnormal replication intermediates (Lonn *et al*., 1990; Poot *et al*., 1992). Taken together, these data argue for a role for RecQ helicases at the replication fork.

Unlike components of the MCM complex—the presumed helicase of the growing fork (Labib and Diffley, 2001)—RecQ proteins are not essential for cell growth in the budding and fission yeasts and are therefore not likely to be involved in processive DNA synthesis. The simultaneous inactivation of Sgs1p and Srs2p, another DNA helicase, induces major growth defects in *S.cerevisiae* (Gangloff *et al*., 2000; McVey *et al*., 2001), and it had been proposed initially that Sgs1p and Srs2p are required for replication fork progression (Lee *et al*., 1999). However, this low viability of *sgs1 srs2* cells is suppressed by the inactivation of the homologous recombination pathway, demonstrating that Sgs1p is not required for processive DNA synthesis, but rather is involved in the suppression of inappropriate recombination in S phase (Gangloff *et al*., 2000).

Interactions between RecQ helicases and components of the DNA recombinational repair machinery further support a role for these enzymes in the coordination of replication and recombination. In mammals, BLM is a component of BASC, a genome surveillance complex coordinating multiple recombination and repair activities (Wang *et al*., 2000). Like BLM, the yeast Sgs1 helicase interacts with Rad51p and Top3p (Bennett *et al*., 2000; Wu *et al*., 2000, 2001). Moreover, Sgs1p functions as a sensor in the intra-S phase checkpoint, potentially linking DNA recombination to cellular surveillance mechanisms (Cobb *et al*., 2002; Kolodner *et al*., 2002).

The partial complementation of the hyper-recombination phenotype of the yeast *sgs1*Δ mutant by the expression of either BLM or WRN suggests that their function has been conserved from yeast to human (Yamagata *et al*., 1998). However, RecQ activity seems to be dispensable for S phase progression in yeast, and *sgs1*Δ cells grow with wild-type kinetics. In contrast, cells derived from BS and WS patients display a prolonged S phase, with slow fork progression and accumulation of abnormal replication intermediates (Hand and German, 1975; Lonn *et al*., 1990;
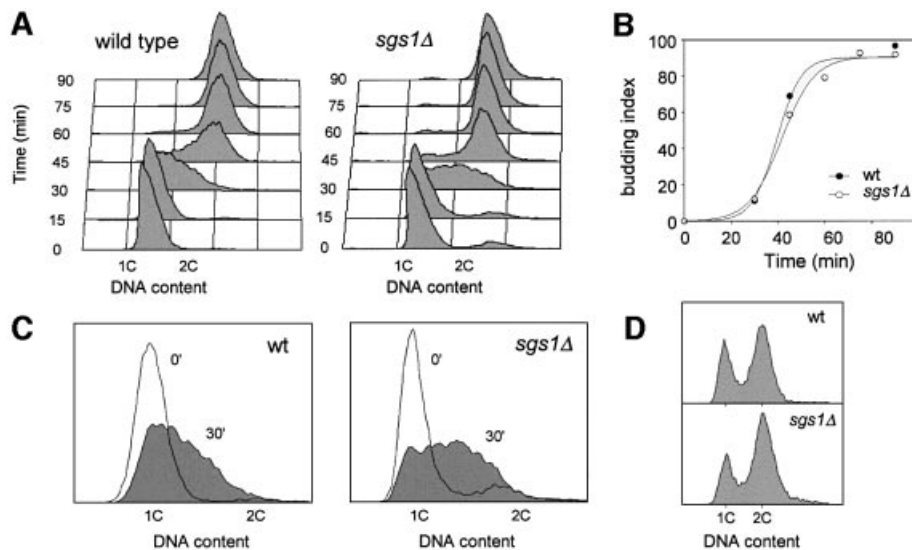
Fig. 1. S phase progression is accelerated in *sgs1Δ* cells. (A–C) Wild-type (E1000) and *sgs1Δ* (E1245) cells were arrested in G_1 with α-mating factor and released synchronously into S phase. (**A**) Samples were collected at the indicated times after release and DNA content was analyzed by flow cytometry. (**B**) Budding index. (**C**) Flow cytometry profiles of wild-type and *sgs1Δ* cells collected at t_0 (G_1) and $t_{30\ min}$ (early S). The proportion of the area of the $t_{30\ min}$ profile extending beyond the G_1 peak is 42% larger in *sgs1Δ* mutants than in wild-type cells. (**D**) Flow cytometry analysis of exponentially growing wild-type and *sgs1Δ* cells. The proportion of G_2/M cells is 40% ($\pm$ 6%) higher in the *sgs1Δ* mutant.
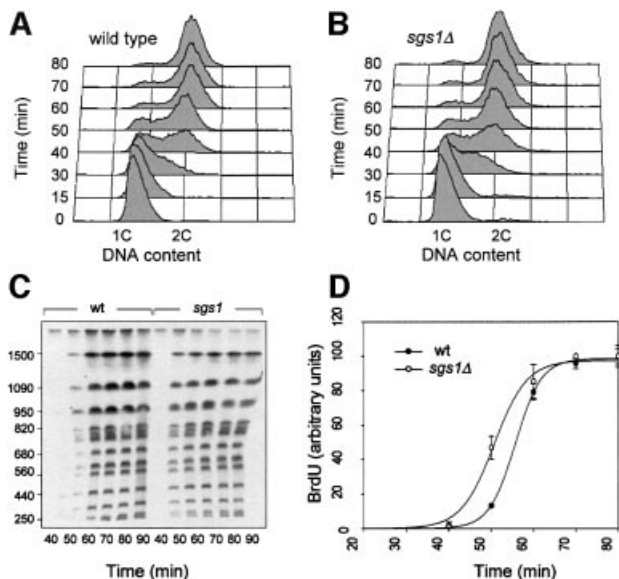
Fig. 2. The Sgs1p helicase is not required for the completion of DNA replication. Wild-type (E1000) and *sgs1Δ* (E1245) cells were released synchronously from an α-factor block in medium supplemented with 400 µg/ml BrdU and harvested at the indicated times. (**A** and **B**) Analysis of DNA content by flow cytometry. (**C**) Genomic DNA prepared from cells embedded in low-melting agarose plugs was separated by PFGE. The amount of BrdU incorporated in fully replicated chromosomes was quantitated as described in Materials and methods. (**D**) Kinetics of the re-emergence of fully replicated chromosomes in wild-type and *sgs1Δ* cells. Relative BrdU incorporation was quantitated for six representative chromosomes. Error bars indicate standard deviation.

Poot *et al*., 1992). Whether this difference reflects a specialization of RecQ function in higher eukaryotes or whether it indicates that the same function is differentially required for S phase progression in yeast and mammals remains to be addressed.

Here, we have monitored S phase progression in wild-type and *sgs1Δ* yeast cells using flow cytometry, pulsed-field gel electrophoresis (PFGE), two-dimensional gel analysis and a new technique called DNA combing, which allows the analysis of replication on single DNA molecules. We show that *sgs1Δ* cells traverse S phase significantly faster than wild-type cells because forks are moving more rapidly. In contrast, we find that completion of DNA replication is impaired at ribosomal DNA (rDNA), which contains a high density of replication fork barriers. These data suggest that Sgs1p is required neither for elongation nor for the decatenation of newly replicated chromosomes, but plays an important role in the suppression of illegitimate recombination occurring at stalled replication forks.

Results

The Sgs1 helicase slows down progression through S phase

To examine the effect of *SGS1* inactivation on DNA replication, congenic wild-type (E1000) and *sgs1Δ* (E1245) cells were arrested in G_1 with α-factor and released synchronously into the cell cycle. Cells were collected every 15 min from release, and DNA content was monitored by flow cytometry (Figure 1A). The appearance of buds, indicative of entry into the cell cycle, was scored (Figure 1B). Surprisingly, the DNA content appeared to increase faster in the *sgs1Δ* mutant than in wild-type cells (Figure 1A; compare time points 30 and 45 min), although both strains passed START synchronously (Figure 1B). In order to quantify this difference, the area of S phase peaks at $t = 30$ min was integrated in four independent experiments (example in Figure 1C). The DNA content per haploid cell was 36% ($\pm$ 8%) greater at this time in the absence of Sgs1 helicase. Interestingly, flow cytometry analysis of log phase cultures grown on synthetic complete medium at 25°C revealed that the proportion of cells with
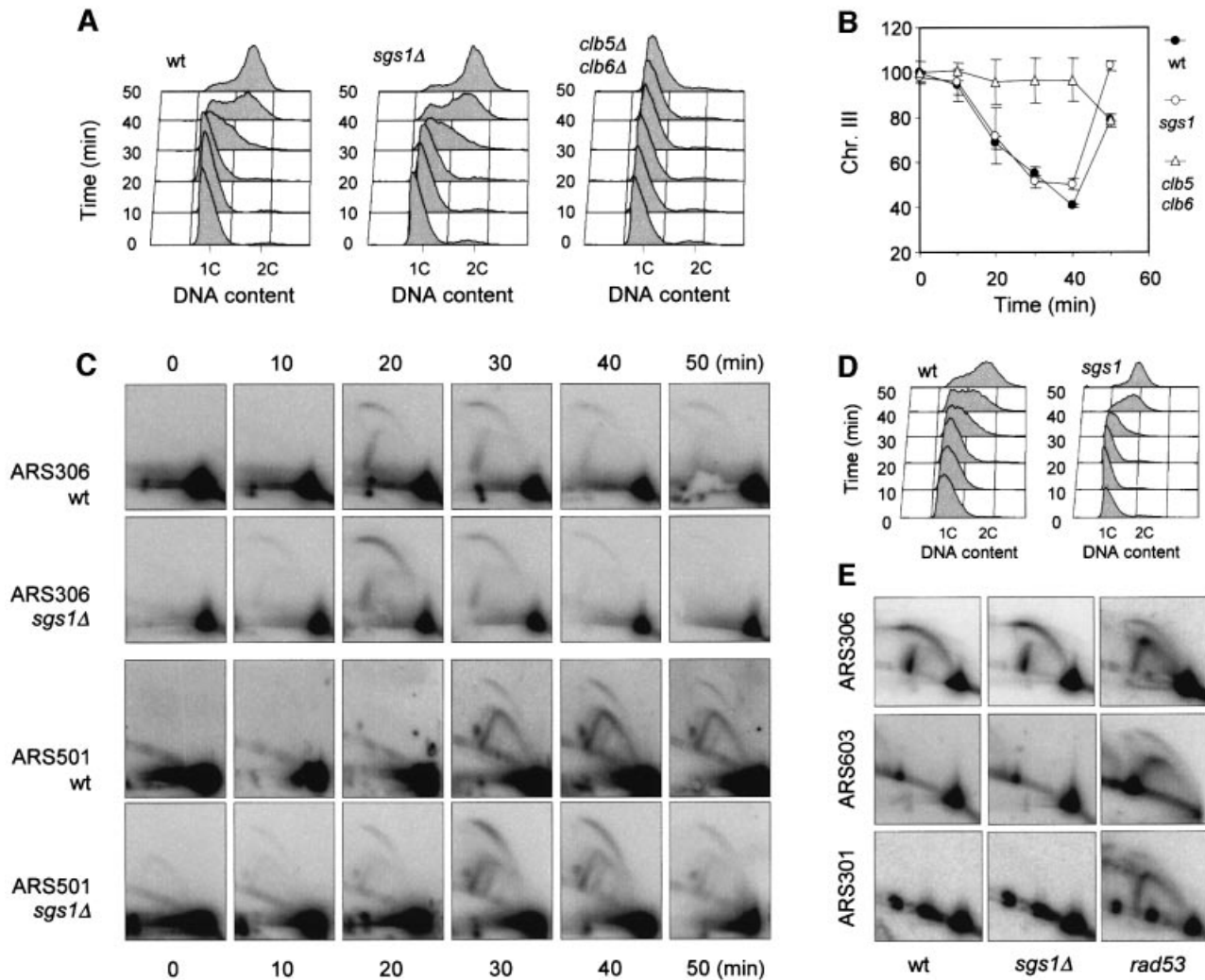
Fig. 3. Time of origin firing in wild-type and *sgs1*Δ cells. (**A**) Wild-type (E1000), *sgs1*Δ (E1245) and *clb5*Δ *clb6*Δ (E742) cells were arrested in G$_1$ with α-factor. Cells were collected every 10 min after degradation of α-factor, and DNA content was analyzed by flow cytometry. (**B**) The electrophoretic mobility of chromosome III was analyzed by PFGE, and DNA content was quantitated with a phosphoimager. (**C**) Analysis of the timing of origin activation in wild-type (E1000) and *sgs1*Δ (E1245) cells. Cells were released synchronously into S phase from an α-factor block, and samples were taken at the indicated times after release. Neutral/neutral two-dimensional gel analysis (Brewer and Fangman, 1987) was used to monitor initiation at the early replication origin *ARS306* and at the late origin *ARS501*. (**D**) Flow cytometry profiles of the samples analyzed in (C). (**E**) Two-dimensional gel analysis of initiation at the early origin *ARS306*, the late origin *ARS603* and the dormant origin *ARS301* in wild-type (E1000), *sgs1*Δ (E1245) and *rad53-11* (E1019) cells released into S phase for 90 min in the presence of 200 mM HU.

a 2C DNA content is ~40% higher in the *sgs1*Δ mutant (Figure 1D), indicating that although *sgs1*Δ cells progress faster through S phase, they accumulate transiently in G$_2$ or M phase.

Sgs1 is not required for the termination of DNA replication

Sgs1p binds topoisomerases II and III, which are required for the decatenation of late replication intermediates (Gangloff *et al.*, 1994; Watt *et al.*, 1995). The elevated rate of missegregation in *sgs1*Δ cells could therefore reflect their inability to resolve newly replicated chromosomes. To test this hypothesis, we have monitored the completion of DNA replication in wild-type and *sgs1*Δ cells by PFGE. This approach is based on the fact that incompletely replicated chromosomes cannot be separated by PFGE (Hennessy *et al.*, 1991). Thymidine kinase-expressing (TK$^+$) cells, which are capable of incorporating bromodeoxyuridine (BrdU) (Lengronne *et al.*, 2001), were

released synchronously from a G$_1$ arrest in complete medium supplemented with BrdU and were collected at regular intervals throughout the cell cycle (Figure 2A and B). Chromosomal DNA from wild-type and *sgs1*Δ cells was subsequently purified in agarose plugs and was separated by PFGE. As expected, BrdU-labeled DNA did not enter the gel until the end of S phase (Supplementary figure 1 available at *The EMBO Journal* Online). The re-emergence of fully replicated chromosomes was quantitated after transfer to a nitrocellulose membrane and detection of BrdU (Figure 2C and D). Interestingly, the bulk of chromosomal DNA re-entered the gel 5–10 min earlier in *sgs1*Δ cells, which represents approximately a quarter of the length of S phase in wild-type cells. This faster completion of DNA replication is reminiscent of the accelerated S phase progression observed by flow cytometry. Moreover, it shows that Sgs1p is not required for the timely resolution of late replication intermediates. However, the transient accumu-
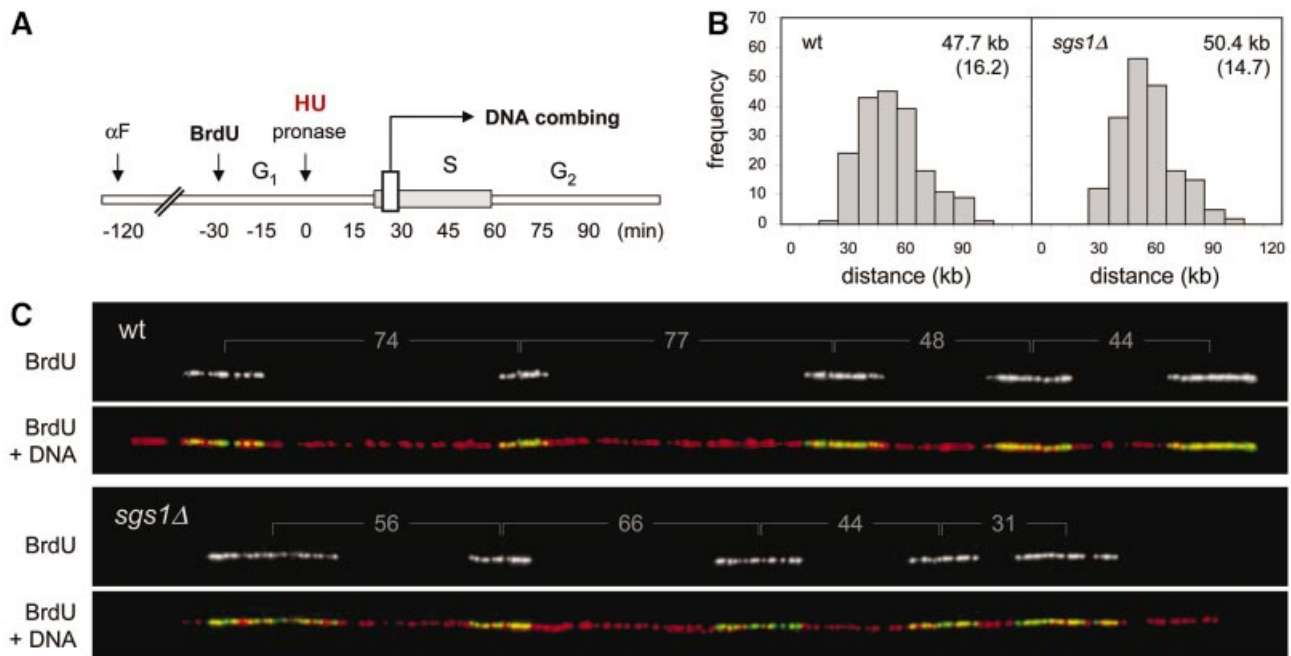
Fig. 4. Single molecule analysis of the initiation rate in wild-type and *sgs1Δ* cells. (**A**) Wild-type (E1000) and *sgs1Δ* (E1245) cells were synchronized in G₁ with α-factor and were released for 90 min into S phase in the presence of BrdU and 200 mM HU, to block elongation. (**B**) DNA fibers were combed on silanized coverslips and the center to center distances between adjacent BrdU tracks were measured as described in Materials and methods. For each strain, a total of 9.4 Mb of DNA was analyzed. Means and standard deviations are indicated. (**C**) Examples of DNA fibers. The center to center distance between BrdU tracks is indicated in kbp. Green, BrdU; red, DNA.

lation of cells with a 2C DNA content (Figure 1D) suggests that, although the bulk of genomic DNA is rapidly replicated, a significant proportion of the cells may experience problems in separating their chromosomes.

Faster S phase progression in sgs1Δ cells is not due to a premature G₁/S transition

To check whether the faster completion of DNA replication in *sgs1Δ* cells is due to a premature entry into S phase, we have analyzed the kinetics of origin activation on a specific chromosome (chromosome III) in wild-type and *sgs1Δ* cells by PFGE (Figure 3A and B). In both strains, chromosome III is excluded progressively from the gel 10 min after release, indicating that cells enter synchronously into S phase. In contrast, chromosome III mobility decreased only after 40 min in *clb5Δ clb6Δ* cells (Figure 3B), which is consistent with the fact that S phase is delayed by 30 min in this mutant (Schwob and Nasmyth, 1993). Analysis of other yeast chromosomes gave the same result (data not shown). To confirm this observation using an independent approach, we have examined the time of ARS306 activation in wild-type and *sgs1Δ* cells by two-dimensional gel electrophoresis (Brewer and Fangman, 1987). ARS306 is the first origin to fire on chromosome III. As shown in Figure 3C, it is activated ~20 min after release from the α-factor arrest in both strains. However, we noticed that replication intermediates disappeared more rapidly in the *sgs1Δ* mutant, suggesting either that initiation is more synchronous in this population of cells or that forks progress faster in the absence of Sgs1p. Interestingly, the time of ARS306 activation corresponds exactly to the time of the exclusion of chromosome III from the gel. However, this chromo-

some completed replication faster in *sgs1Δ* cells (Figure 3B), which is consistent with the data shown in Figure 2D. Taken together, these results indicate that the faster replication in *sgs1Δ* cells is not due to premature entry into S phase.

The mechanisms delaying initiation at late origins are functional in sgs1Δ cells

We next analyzed the time of initiation at the subtelomeric late origin ARS501 and the internal late origin ARS603 using two-dimensional gel electrophoresis. We found that these origins are activated ~10 min after the early origin ARS306 in both strains (Figure 3C, and data not shown). Although late origins appeared to fire slightly earlier in a fraction of *sgs1Δ* cells, this suggests that the timing of origin activation is not significantly affected by the absence of Sgs1p. Late origins are normally repressed by the replication checkpoint in the presence of hydroxyurea (HU) (Santocanale and Diffley, 1998), but fire in *rpd3Δ* cells exposed to HU, presumably because late origin initiation is advanced in these cells (O.Aparicio, unpublished results). To check whether this is also the case for *sgs1Δ* cells, we analyzed the activity of the late origin ARS603 and of the dormant origin ARS301 by two-dimensional gel electrophoresis. As shown in Figure 3E, these origins are repressed in wild-type and in *sgs1Δ* cells, but fire in the *rad53-11* mutant (Santocanale and Diffley, 1998; Santocanale *et al.*, 1999). Similarly, ARS501 is inactive in *sgs1Δ* cells exposed to HU (J.Cobb and S.Gasser, submitted). Taken together, these results indicate that the mechanisms controlling the activation of late replication origins are active in *sgs1Δ* cells.
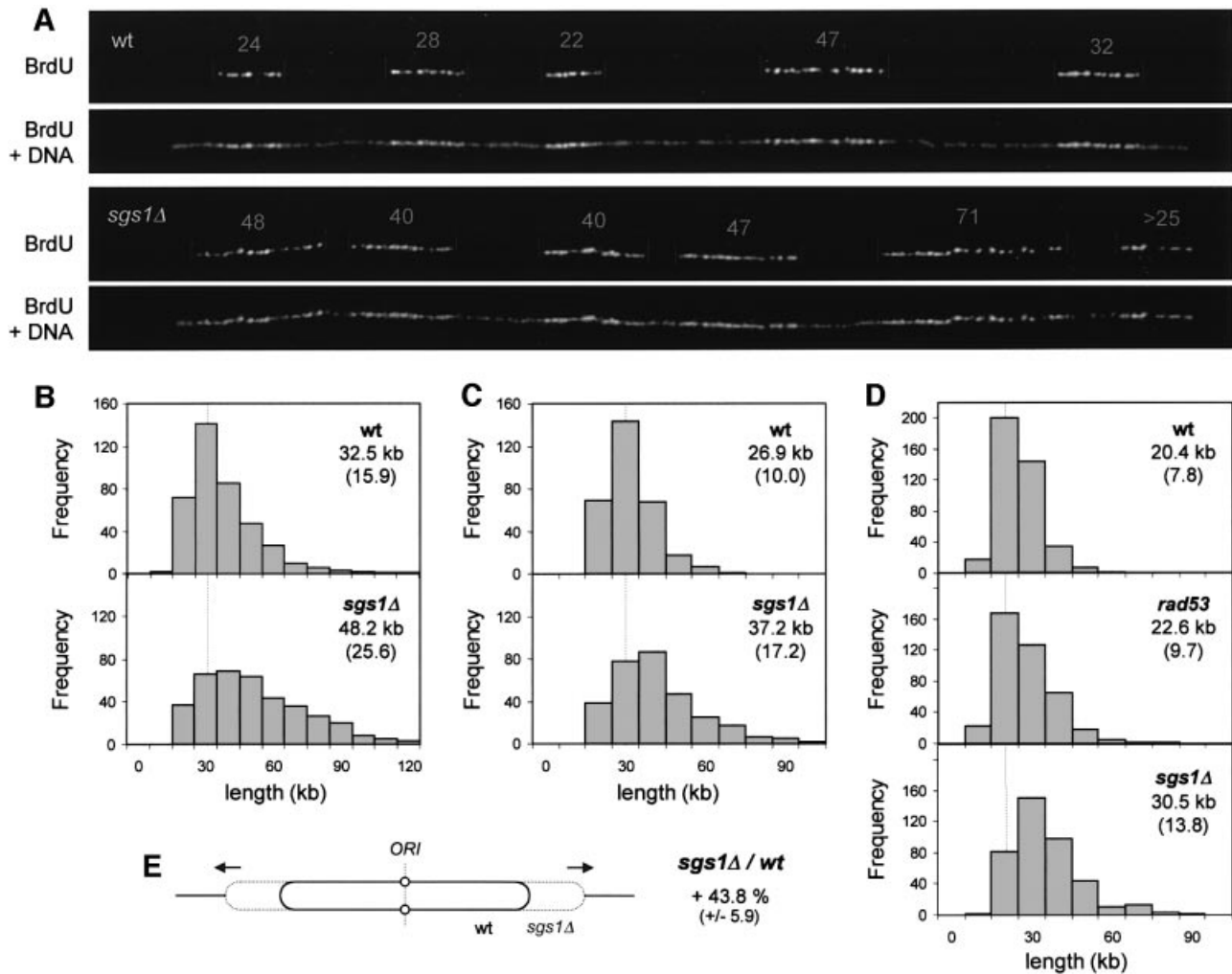
Fig. 5. Replication forks progress faster in the absence of Sgs1p. (**A**) Wild-type (E1000) and *sgs1*Δ (E1245) cells were released synchronously from an α-factor block in BrdU-supplemented medium as described in Figure 4, but this time HU was omitted. Samples were collected in mid-S phase (30 min after release) and BrdU tracks were detected after DNA combing. The length of BrdU tracks (kb) is indicated for two representative molecules. (**B**) Size distribution of BrdU tracks in wild-type and *sgs1*Δ cells. For each strain, ~400 signals, encompassing >15 Mb in total, were measured. (**C**) Early log phase cultures of wild-type and *sgs1*Δ cells were labeled for 25 min with 400 μg/ml BrdU. The distribution of BrdU track length was determined as described above. For each strain, ~300 BrdU signals were measured (>10 Mb in total). (**D**) Early log phase cultures of wild-type, *sgs1*Δ and *rad53-11* (E1019) cells were labeled for 22 min and the distribution of BrdU tracks length was determined (400 BrdU signals for each strain). (**E**) The analysis of four independent sets of experiments indicates that BrdU tracks are reproducibly longer (43.8 ± 5.9%) in the absence of the Sgs1 helicase.

The accelerated S phase in sgs1Δ cells is not due to a higher frequency of initiation

Several lines of evidence indicate that most of the yeast replication origins are active in only a fraction of the cell cycles. In *sgs1*Δ cells, an overall increase of this initiation rate could explain their faster progression through S phase. To address this possibility, we have compared the density in active origins in wild-type and *sgs1*Δ cells using BrdU incorporation and dynamic molecular combing (Michalet *et al.*, 1997). Cells were released synchronously into S phase in the presence of BrdU to label initiation sites, and elongation was blocked with the addition of HU (Figure 4A). Chromosomal DNA was prepared in agarose plugs to avoid shearing and was combed on silanized coverslips. The sites of BrdU incorporation were subsequently detected along individual molecules with fluorescent antibodies, and DNA fibers were counterstained with an anti-guanosine antibody (Figure 4C). Since combed DNA fibers are stretched uniformly (Michalet *et al.*, 1997),

the statistical analysis of center to center distances between adjacent BrdU tracks provides an accurate indication of origin density (Lengronne and Schwob, 2002; Pasero *et al.*, 2002). Here, the analysis of ~10 Mb of DNA per strain revealed that active origins are not more abundant in *sgs1*Δ cells than in wild-type cells (Figure 4B). It is worth noting that initiation at late and dormant origins is not detected in this assay. However, we confirmed by two-dimensional gel electrophoresis that there is no promiscuous activation of dormant origins such as ARS301 in untreated *sgs1*Δ cells (data not shown). We therefore believe that the faster S phase of *sgs1*Δ cells is not due to an overall increase of origin activity.

Progression of replication forks is accelerated in sgs1Δ cells

An alternative explanation for the faster S phase in *sgs1*Δ cells could be that forks are moving more rapidly in the absence of the Sgs1p helicase. In the experiment described

above, we found that BrdU tracks were on average 22% longer in HU-arrested *sgs1Δ* cells (wild type, 18.4 ± 9.2 kb; *sgs1Δ*, 22.5 ± 12.2 kb). To examine whether BrdU tracks are also longer in *sgs1Δ* cells in the absence of drug, wild-type and *sgs1Δ* cells were released synchronously from G_1 and genomic DNA was isolated from cells harvested in mid-S phase ($t = 30$ min). Individual chromosomes were combed, and newly replicated regions were visualized using anti-BrdU antibodies (Figure 5A). The analysis of a large number of BrdU tracks (~400 per strain) revealed that newly replicated stretches are 48% longer in *sgs1Δ* cells (Figure 5B). These data suggest that the rate of elongation is faster in *sgs1Δ* cells.

To ensure that longer BrdU tracks in *sgs1Δ* cells are not due to an artifact of synchronization, exponentially growing cultures of wild-type and *sgs1Δ* cells were pulse labeled for 25 min with BrdU and the length of newly replicated segments was measured by DNA combing. Again, we found a 40–50% increase of the mean BrdU track length in *sgs1Δ* cells (Figure 5C and D). The combination of four independent experiments confirmed that BrdU tracks are 44% (± 6%) longer in *sgs1Δ* cells (Figure 5E). Taken together, these results indicate that the Sgs1p helicase is required neither for the progression of the replication fork nor for the timely resolution of replicated chromosomes. Instead, we find that Sgs1p slows down the global rate of elongation.

The checkpoint kinase Rad53 does not slow down fork progression

In response to fork arrest induced by genotoxic drugs, yeast cells activate the checkpoint kinases Mec1p and Rad53p, which in turn block late origin firing and stabilize stalled forks (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Sogo *et al.*, 2002). Since Sgs1p is required for the activation of Rad53p in HU-arrested cells, at least in Rad24-deficient cells (Frei and Gasser, 2000), we asked whether Sgs1p acts through Rad53p to slow down fork progression in a normal S phase. If this were the case, a *rad53* mutant should also display an accelerated fork progression in our assay. To test this possibility, we measured the length of BrdU tracks in congenic wild-type (E1000), *sgs1Δ* (E1245) and *rad53-11* (E1019) cells pulse labeled in mid-log phase as described above. We found that BrdU tracks were roughly identical in wild-type and *rad53* cells, while they were again 50% longer in *sgs1Δ* cells (Figure 5D). These data indicate therefore that Rad53p does not regulate the rate of elongation in unchallenged growth conditions.

Completion of DNA replication is delayed at the rDNA locus of sgs1Δ cells

Sgs1-deficient cells are sensitive to sublethal doses of HU, presumably because they fail to stabilize stalled forks (J.Cobb and S.Gasser, submitted). We therefore reasoned that a locus naturally containing a high density of replication fork barriers, namely the rDNA array (Brewer and Fangman, 1988; Linskens and Huberman, 1988), should be more prone to replication fork collapse than bulk genomic DNA. To test this possibility, the rDNA array was excised by restriction digestion (Supplementary figure 2A), and completion of rDNA replication was monitored by PFGE. In wild-type cells, we found that the
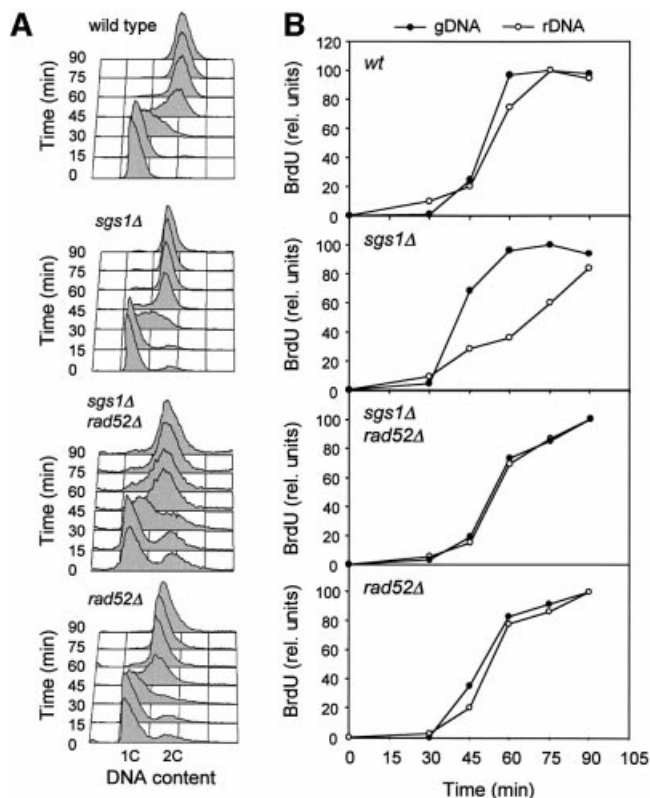

Fig. 6. Altered electrophoretic mobility of the rDNA array in *sgs1Δ* cells. Wild-type (E1000), *sgs1Δ* (E1245), *rad52Δ* (E1384) and *sgs1Δ rad52Δ* (E1382) cells were released synchronously into S phase in the presence of 400 μg/ml BrdU, and samples were collected at the indicated times. (**A**) Analysis of DNA content by flow cytometry. (**B**) Comparative analysis of the electrophoretic mobility of genomic DNA (gDNA) and rDNA in the four different strains. The re-emergence of fully replicated DNA molecules was quantitated as described in Figure 2.

rDNA array re-entered the gel slightly after genomic DNA, suggesting that branched structures persist for a longer period of time at this locus (Figure 6B). Interestingly, this delay was exacerbated in *sgs1Δ* cells, the rDNA array re-entering the gel 20–30 min after the other chromosomes (Figure 6B). The same delay was observed when the mobility of rDNA arrays from wild-type and *sgs1Δ* cells were compared with each other (Supplementary figure 2B and C), or when chromosome XII, which bears the rDNA array, was compared with other chromosomes (Supplementary figure 2F). Interestingly, a similar alteration of chromosome XII mobility, which bears the rDNA array, has been reported recently in *sgs1-ts slx4Δ* double mutants (Kaliraman and Brill, 2002).

Wild-type and sgs1Δ cells display similar RFB and ARS activities

The rDNA of *S.cerevisiae* contains ~150 ARS elements, but only 20% of these potential replication origins are used every cell cycle. Forks moving leftward from the rDNA origin are arrested by a structure called a replication fork barrier (RFB), which persists until it merges with rightward-moving forks (Brewer and Fangman, 1988; Linskens and Huberman, 1988). To check if the altered mobility of
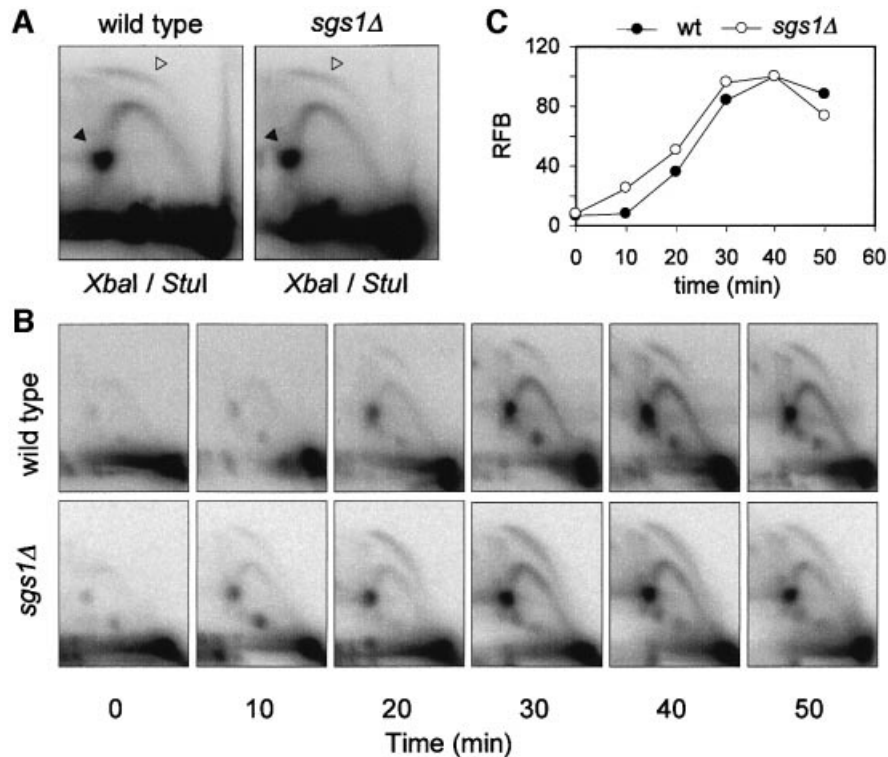
Fig. 7. Analysis of rDNA replication and RFB activity by two-dimensional gel electrophoresis. (**A**) Genomic DNA was extracted from exponentially growing wild-type (E1000) and *sgs1Δ* (E1245) cells, and a *Stu*I–*Xba*I rDNA fragment (3.5 kb) centered around the origin was analyzed by two-dimensional gel electrophoresis. Quantitation of bubble arc (open arrowhead) and RFB signals (replication fork barrier, filled arrowhead) with a phosphoimager did not reveal any significant difference between the two strains in three independent experiments. (**B**) Two-dimensional gel analysis of rDNA ARS and RFB activity in wild-type and *sgs1Δ* cells released synchronously into S phase (see Figure 3D for the corresponding flow cytometry profiles). (**C**) Quantitation of RFB signals in wild-type and *sgs1Δ* cells.

the rDNA in *sgs1Δ* cells was due to a delayed initiation at this locus or to a persistence of stalled forks in late S phase, we have monitored the activity of the rDNA ARS and of the RFB by two-dimensional gel electrophoresis. The quantitation of replication intermediates in three independent experiments showed that origin activity and RFB signals were virtually identical in exponentially growing wild-type and *sgs1Δ* cells (Figure 7A). To confirm this result, we have analyzed rDNA replication in cells released synchronously in S phase. Bubble arcs were detected throughout the S phase at the rDNA (Figure 7B). Quantitation of RFB signals with a phosphoimager revealed a similar pattern in both strains, although the rDNA replicated slightly earlier in the *sgs1Δ* mutant (Figure 7C; Supplementary figure 3A). Moreover, the amount of stalled forks decreased after 50 min in both strains, and RFB signals were almost undetectable in *sgs1Δ* cells 20 min later (Supplementary figure 3B). We therefore assume that the altered electrophoretic mobility of the rDNA in *sgs1Δ* cells is not due to a replication initiation defect nor to the persistence of stalled replication forks.

The altered mobility of the rDNA array is due to homologous recombination

Deletion of the *SGS1* gene increases homologous recombination at rDNA (Gangloff *et al.*, 1994). To check whether the altered electrophoretic mobility of this locus in *sgs1Δ* cells is due to the presence of unresolved

recombination intermediates, we have inactivated the homologous recombination pathway in these cells. Interestingly, the migration of the rDNA array was restored in *rad52Δ sgs1Δ* cells (Figure 6B). In the absence of Sgs1p, the abnormal mobility of the rDNA array in late S phase is therefore likely to be attributable to the persistence of unresolved recombination intermediates. It is worth noting that we were unable to detect these intermediates on two-dimensional gels (Figure 7B). In contrast, a strong X-spike, corresponding to Holliday junctions, has been reported at the rDNA of polymerase α and δ mutants (Zou and Rothstein, 1997). We assume that these intermediates are less abundant in *sgs1Δ* cells, or correspond to different recombination intermediates.

ERCs accumulate faster in young *sgs1Δ* cells, but not in aging cells

Intra-chromatid recombination at rDNA generates extra-chromosomal rDNA circles (ERCs), which have been implicated in replicative senescence (Sinclair and Guarente, 1997). ERCs are present at one or two copies per cell in log phase cultures, but accumulate to very high levels in aging cells (Sinclair and Guarente, 1997; Ivessa *et al.*, 2000). Here, we have examined ERC levels in *sgs1Δ* cells, as a marker of rDNA recombination, regardless of their role in replicative senescence. We measured a 3.7-fold (± 0.8) increase of ERC levels in young *sgs1Δ* cells (Figure 8A), which is consistent with the 5-fold increase of rDNA recombination measured in these cells with other

techniques (Gangloff *et al.*, 1994; Defossez *et al.*, 1999; Heo *et al.*, 1999; Hoopes *et al.*, 2002; Merker and Klein, 2002).

The faster accumulation of ERCs in old *sgs1Δ* cells was evoked to explain their premature aging phenotype (Sinclair and Guarente, 1997). However, this model has been challenged in recent studies showing that ERCs are not more abundant in old *sgs1Δ* cells than in old wild-type cells (Heo *et al.*, 1999; McVey *et al.*, 2001; Kaliraman and Brill, 2002). To reconcile our observations with these reports, we have examined ERC levels in wild-type and *sgs1Δ* cultures enriched in old mother cells (seven generations). As expected, we observed a dramatic increase of ERC levels in old wild-type and *sgs1Δ* cells compared with young cells (Figure 8B). However, with the exception of a slow migrating form detected above the rDNA array, the amount of ERC species appeared to be similar in old wild-type and *sgs1Δ* cells (Figure 8B, asterisked arrowhead). This observation is consistent with earlier reports indicating that ERCs are not responsible for the accelerated aging phenotype of *sgs1Δ* cells (McVey *et al.*, 2001).

Accumulation of ERCs in sgs1Δ cells is Rad52 and Fob1 dependent

Since deletion of the *RAD52* gene relieves the altered electrophoretic mobility of the rDNA array in *sgs1Δ* cells (Figure 6), we checked whether this would also suppress the accumulation of ERCs. We found that the inactivation of *RAD52*, but not of *RAD51*, completely suppressed the formation of ERCs in *sgs1Δ* cells (Figure 8C; Supplementary figure 4A), which is consistent with the fact that Rad51p is not required for homologous recombination at the rDNA (Zou and Rothstein, 1997). Moreover, deletion of the *FOB1* gene, which is essential for RFB activity (Kobayashi and Horiuchi, 1996; Defossez *et al.*, 1999), almost completely suppressed the formation of ERCs in *sgs1Δ* cells (Figure 8C) and relieved the altered electrophoretic mobility of the rDNA (Supplementary figure 4B). Taken together, these data indicate that rDNA replication is delayed in *sgs1Δ* cells because of hyper-recombination occurring at replication forks arrested at RFBs.

Discussion

RecQ DNA helicases are important for the maintenance of genome integrity and have been implicated in several aspects of DNA replication. However, the nature of their contribution to DNA synthesis has remained unclear. Here, we report that yeast cells progress faster through S phase in the absence of Sgs1p. We have used two-dimensional gels and PFGE mobility assays to show that this is not due to a premature entry into S phase, nor to the promiscuous activation of dormant replication origins. To investigate the potential causes of this accelerated S phase, we have analyzed the replication of single DNA molecules by dynamic molecular combing. We found that this faster replication is not due to changes in origin usage but rather to a faster progression of replication forks, which was best demonstrated in experiments with unsynchronized cells. It should be noticed that late origins appeared to fire slightly earlier in *sgs1Δ* cells. Although this effect is much stronger
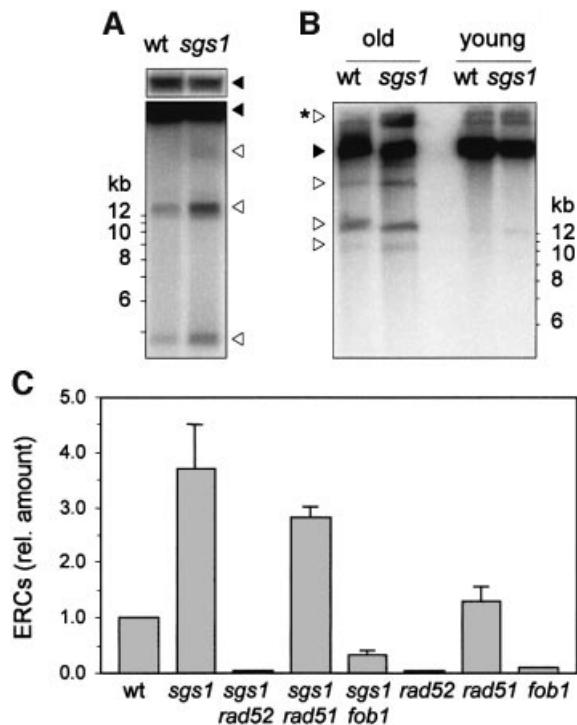
Fig. 8. Quantitation of extrachromosomal rDNA circles in *sgs1Δ* cells. (**A**) Genomic DNA prepared from log phase wild-type (E1000) and *sgs1Δ* (E1245) cultures was separated by conventional gel electrophoresis. ERCs (empty arrowheads) were detected by Southern blotting and hybridization with a radioactive rDNA probe. A shorter exposure of the signal corresponding to the rDNA array is shown (filled arrowheads). (**B**) Detection of ERC species in young and old *sgs1Δ* cells. Cultures of wild-type (E1000) and *sgs1Δ* (E1245) cells were enriched in old mother cells (seven generations) as described (Sinclair and Guarente, 1997). (**C**) ERC levels in *sgs1Δ* (E1245), *sgs1Δ rad52Δ* (E1382), *sgs1Δ rad51Δ* (E1450), *sgs1Δ fob1Δ* (E1457), *rad52Δ* (E1384), *rad51Δ* (E1059) and *fob1Δ* (E1740) were quantitated with a phosphoimager and are expressed relative to the amount of ERCs in wild-type cells.

in *rad53* mutants, it does not affect the length of the S phase (Shirahige *et al.*, 1998) nor the elongation rate in *rad53* cells (this work). We therefore assume that late origins fire slightly earlier in *sgs1Δ* cells because S phase is shorter, the primary cause for this accelerated S phase being the faster progression of replication forks.

Why DNA replication forks are moving more rapidly in the absence of Sgs1p is an intriguing issue. Since Sgs1p interacts with several components of the replication machinery and co-localizes with sites of ongoing DNA synthesis (Cobb *et al.*, 2002), it could be permanently associated with the replication fork, hindering its progression via an unknown mechanism. Alternatively, Sgs1p could only act at the fork when it encounters an obstacle, such as a lesion of DNA or a replication pause site. Programmed pause sites have been found throughout the yeast genome (Deshpande and Newlon, 1996; Wang *et al.*, 2001; Cha and Kleckner, 2002), and Sgs1p could stabilize forks traversing these sites in order to prevent illegitimate recombination. In *sgs1Δ* mutants, replication forks may progress faster through these sites, leading to a concomitant increase of the elongation rate and of genomic instability. We currently favor this second model, and experiments are under way to test it.

Genetic evidence indicates that Sgs1p acts together with Rad24p as sensor proteins to activate S phase checkpoints (Frei and Gasser, 2000; Myung and Kolodner, 2002). To test whether Sgs1p acts through Rad53p to regulate fork progression in normal growth conditions, we have measured the rate of elongation in *rad53-11* cells by DNA combing. We found that Rad53p alone does not slow down fork progression in a normal S phase, as is the case for cells exposed to DNA damage (Tercero and Diffley, 2001). We therefore propose that Sgs1p modulates fork progression directly, independently of its checkpoint function.

The faster progression of replication forks in RecQ-deficient yeast cells contrasts strikingly with the slower rate of elongation reported previously in human BS and WS cells (Lonn *et al.*, 1990; Poot *et al.*, 1992). This discrepancy suggests that the function of RecQ helicases may have diverged during evolution. Alternatively, it could reflect structural differences between yeast and mammalian chromosomes. Indeed, the genome of *S.cerevisiae* is rather AT rich and is almost devoid of heterochromatin. In contrast, chromosomes of higher eukaryotes contain complex chromatin structures and a high frequency of repeated sequences, which are prone to homologous recombination. Moreover, mammalian genomes contain GC-rich repetitive elements, which can adopt alternative DNA structures such as hairpins or G-quartets. These structures are resolved efficiently by BLM, WRN and Sgs1p helicases *in vitro* (Sun *et al.*, 1998, 1999; Fry and Loeb, 1999). In RecQ mutants, they would impede the progression of replication forks and generate genomic instability (Hyrien, 2000; Kamath-Loeb *et al.*, 2001).

In the yeast genome, most of the heterochromatin and the G-rich sequences are found at telomeres and at the ribosomal gene array. Sgs1p is required for telomere maintenance in telomerase-deficient cells (Huang *et al.*, 2001; Johnson *et al.*, 2001). Here, we show that the structure of the rDNA array of *sgs1Δ* cells is altered uniquely in a replication-dependent manner. This is consistent with a recent report showing a similar alteration in an *sgs1 slx4* double mutant (Kaliraman and Brill, 2002). Furthermore, we observed an accumulation of ERCs in young *sgs1Δ* cells, recapitulating their increased rate of rDNA recombination (Gangloff *et al.*, 1994). Both the altered mobility of the rDNA array and the accumulation of ERCs were totally suppressed by the inactivation of the homologous recombination pathway in a *rad52Δ* mutant. We therefore propose that in the absence of Sgs1p helicase, unprotected replication increases genomic instability at the rDNA.

The rDNA array contains a high density of replication fork barriers (Brewer and Fangman, 1988; Linskens and Huberman, 1988), which represent a potential source of homologous recombination. We have used two-dimensional gel analysis to compare the abundance of forks arrested at RFBs in wild-type and *sgs1Δ* cells. No significant differences were detected between the two strains. Moreover, we found that RFB signals disappear almost 20 min before the resolution of intermediates, altering the electrophoretic mobility of the rDNA in *sgs1Δ* cells. This indicates that RFBs *per se* are not responsible for the abnormal electrophoretic mobility of the rDNA array in *sgs1Δ* cells. To check whether RFBs alter the mobility of rDNA via the formation of recombination intermediates, we have deleted the *FOB1* gene in *sgs1Δ* cells. Suppressing RFB activity in these cells relieved this altered PFGE mobility and strongly reduced the formation of ERCs. However, unlike *sgs1Δ rad52Δ* mutants, low levels of ERCs were detected in RFB-deficient *sgs1Δ* cells. Fork arrest must therefore occur at other sites in *fob1Δ sgs1Δ* cells, as is the case for *rrm3* mutants (Ivessa *et al.*, 2000).

What is the primary source of homologous recombination in *sgs1Δ* cells? A branched structure, sometimes referred to as a 'chicken foot', has been proposed to form by reversion of stalled forks and to induce a DSB (Klein and Kreuzer, 2002). Such a structure is found frequently in *rad53* cells exposed to HU (Sogo *et al.*, 2002) and is resolved efficiently *in vitro* by Sgs1p (Bennett *et al.*, 1999). However, recent genetic evidence argues against a role for DSBs in the genomic instability of *sgs1Δ* cells (Fabre *et al.*, 2002). According to this report, Sgs1p would instead prevent the formation of single-stranded DNA (ssDNA) at stalled forks, on which recombination is initiated. ssDNA is not normally found at RFB-arrested forks in wild-type cells (Gruber *et al.*, 2000), and its presence in *sgs1Δ* cells remains to be demonstrated. Interestingly, a loss of coordination of DNA polymerases during fork arrest has been observed recently in these cells (J.Cobb and S.Gasser, submitted). This provides a plausible mechanism for the formation of ssDNA in *sgs1Δ* mutants.

In conclusion, our data suggest that forks progress faster in the absence of Sgs1p helicase but are less stable, particularly at regions of the yeast genome containing a high density of replication fork barriers such as the rDNA array. It is worth noting that the replication defects observed specifically at the yeast rDNA are reminiscent of the replication defects observed in human BS and WS cells (Lonn *et al.*, 1990; Poot *et al.*, 1992). We therefore propose that yeast and human RecQ orthologs are equally involved in the protection of genome integrity during DNA replication, but that the complexity of vertebrate genomes makes them more dependent on RecQ helicases to replicate their chromosomes safely.

Materials and methods

Strains and synchronization procedure

Congenic E1000 (*MAT***a**, *ade2-1 trp1-1 can1-100 leu2-3 112 his3-11,15 ura3-1 GAL, ura3::URA3/GPD-TK$_{7x}$*), E1245 (*MAT***a**, *sgs1::LEU2, TK$^+$*), E742 (*MAT***a**, *clb5::HIS3, clb6::LEU2, TK$^+$*), E1019 (*MAT***a**, *rad53-11, TK$^+$*), E1382 (*MAT***a**, *sgs1::LEU2, rad52::TRP1, TK$^+$*), E1384 (*MAT***a**, *rad52::TRP1, TK$^+$*), E1059 (*MAT*α, *rad51::LEU2*), E1450 (*MAT***a**, *rad51::LEU2, sgs1::LEU2, TK$^+$*), E1740 (*MAT***a**, *fob1::URA3, TK$^+$*) and E1457 (*MAT***a**, *sgs1::HIS3, fob1::URA3, TK$^+$*) cells were grown at 25°C in complete synthetic medium. Mid-log phase cultures (5 × 10^6 cells/ml) were arrested for 2.5 h in G$_1$ with 2 μg/ml α-factor and released into S phase with the addition of 50 μg/ml pronase (Calbiochem) in complete synthetic medium. To label newly replicated DNA, 400 μg/ml BrdU (Sigma) was added to the medium 30 min before release. To block elongation, 200 mM HU (Sigma) was added simultaneously to BrdU and cells were collected 90 min after release from the G$_1$ arrest. Cell cycle progression was monitored by flow cytometry (FACScan) as described (Epstein and Cross, 1992).

Pulsed-field gel electrophoresis and quantitation of BrdU incorporation

Yeast cells were embedded in low-melting agarose plugs (5 × 10^7 cells/ ml) and genomic DNA was extracted as described (Lengronne *et al.*,

2001). Yeast chromosomes were separated by PFGE (Gene Navigator, AP Biotech) and transferred to a nitrocellulose membrane (Protran, Schleicher & Schüll). BrdU was detected with a mouse monoclonal antibody (Dako) and two layers of IgG coupled to Alexa 488 (Molecular Probes) as described (Lengronne *et al.*, 2001). The membrane was scanned with a FluorImager and signals were quantitated with ImageQuant (Amersham Biosciences).

Dynamic molecular combing

DNA combing was performed as described (Michalet *et al.*, 1997), with the following modifications. Genomic DNA prepared in agarose plugs (800 ng DNA/plug) was stained with YOYO-1 (Molecular Probes) and resuspended at 150 ng/ml in 50 mM MES pH 5.7 after digestion of the plugs with agarase (Roche). Combed DNA fibers were denatured with 1 M NaOH, and BrdU was detected with a rat monoclonal antibody (Sera Lab) and a secondary antibody coupled to Alexa 488 (Molecular Probes). Since denaturation eliminates YOYO-1 staining, DNA molecules were counterstained with an anti-guanosine antibody (Argene) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). Images were recorded with a Leica DMRA microscope coupled to a CCD camera, and signals were measured with MetaMorph (Universal Imaging Corp.). Physical distances between signals were converted into base pairs (1 pixel = 340 bp) using adenovirus DNA molecules as size standard.

Two-dimensional gel analysis and quantitation of ERC species

Neutral/neutral two-dimensional gel analyses were performed as described (Brewer and Fangman, 1987). To monitor the amount of ERC species, genomic DNA was prepared in agarose plugs to avoid shearing, and was separated on a 0.7% agarose gel in 1× TAE without ethidium bromide for 22 h at 1 V/cm. rDNA species were detected after Southern blotting and hybridization with a 3 kb rDNA probe (Pasero *et al.*, 2002). Quantification of autoradiograms was performed by storage phosphoimaging (Amersham). ERC levels were normalized against the chromosomal rDNA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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