Precocious S-Phase Entry in Budding Yeast Prolongs Replicative State and Increases Dependence Upon Rad53 for Viability

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ABSTRACT

Precocious entry into S phase due to overproduction of G1 regulators can cause genomic instability. The mechanisms of this phenomenon are largely unknown. We explored the consequences of precocious S phase in yeast by overproducing a deregulated form of Swi4 (Swi4-t). Swi4 is a late G1-specific transcriptional activator that, in complex with Swi6, binds to SCB elements and activates late G1-specific genes, including G1 cyclins. We find that wild-type cells tolerate Swi4-t, whereas checkpoint-deficient *rad53-11* cells lose viability within several divisions when Swi4-t is overproduced. Rad53 kinase activity is increased in cells overproducing Swi4-t, indicating activation of the checkpoint. We monitored the transition from G1 to S in cells with Swi4-t and found that there is precocious S-phase entry and that the length of S phase is extended. Moreover, there were more replication intermediates, and firing of at least a subset of origins may have been more extensive in the cells expressing Swi4-t. Our working hypothesis is that Rad53 modulates origin firing based upon growth conditions to optimize the rate of S-phase progression without adversely affecting fidelity. This regulation becomes essential when S phase is influenced by Swi4-t.

PROPER integration of cell cycle transitions with DNA metabolism is crucially important for cell survival and error-free propagation of a cell's genetic material. Cells that are unable to adjust the cell cycle clock upon receiving DNA damage are known to lose viability and/or compromise the fidelity of genetic transmission (Foiani *et al.* 2000). In the absence of any genotoxic treatment, genomic stability can be negatively affected by relaxation of the control over the transition between G1 and S phases. For example, forced entry of some quiescent cells into S phase upon c-*myc* or cyclin E overproduction is known to result in genomic rearrangements (Felsher and Bishop 1999; Mai *et al.* 1999; Spruck *et al.* 1999). Loss of Rb or ectopic expression of cyclin D1 in tissue culture cells can increase gene amplification frequencies (Donehower 1997). In budding yeast, inactivation of the B cyclin inhibitor *SIC1*, which causes earlier activation of the S-phase cyclin/CDK complexes and earlier entry into S phase (Schwob *et al.* 1994), leads to an elevated rate of chromosome loss (Nugroho and Mendenhall 1994). Overexpression of G1 cyclins *CLN1* or *CLN2*, whose levels are rate limiting for the G1-to-S transition, is synthetically lethal with mutations in the DNA damage checkpoint kinase gene *MEC1* and causes an increased level of chromosome loss (Vallen and Cross 1995, 1999). The mechanisms of genomic instability induced by relaxation of control over the G1-to-S transition remain largely unknown. We sought to investigate these mechanisms using budding yeast as a model system.

Swi4 is a late G1-specific transcriptional activator, which, in complex with Swi6, binds to SCB elements (Mendenhall and Hodge 1998) in the promoters of numerous late G1-specific genes (Iyer *et al.* 2001). The activity of the Swi4/Swi6 complex gives rise to the concerted burst of transcription of the G1 cyclin genes *CLN1* and *CLN2* and of a number of other genes, which promote the transition to S phase (Nasmyth and Dirick 1991; Ogas *et al.* 1991). Swi4-t, a C-terminally truncated and stabilized form of Swi4, is unable to interact with Swi6 and cannot form the Swi4/Swi6 complex (Andrews and Moore 1992; Primig *et al.* 1992; Sidorova and Breeden 1993). However, unlike the full-length Swi4 (Baetz and Andrews 1999), the truncated Swi4-t is capable of binding to promoters independently of Swi6 (Andrews and Moore 1992; Primig *et al.* 1992; Sidorova and Breeden 1993), and when overexpressed from the *GAL* promoter (*GAL::SWI4-t*), it deregulates the transcription of target genes (Breeden and Mikesell 1994). Swi4-t can cause a precocious entry into S phase similar to the checkpoint mutant of Rad53 in cells that receive DNA damage in G1 (Sidorova and Breeden 1997).

Rad53, a kinase conserved from yeast to humans, is involved in coordinating DNA metabolism with cell cycle transitions (Weinert 1998; Foiani *et al.* 2000). In the presence of DNA damage in G1 and G2, Rad53 is most likely recruited to the damage recognition and processing complex (Foiani *et al.* 2000) via Mec1-phosphorylated Rad9 (Emili 1998; Sun *et al.* 1998; Vialard *et al.* 1998; Durocher *et al.* 1999). Rad53 is phosphorylated and activated in a Mec1 kinase-dependent manner (Navas

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et al. 1996; Sanchez *et al.* 1996; Sun *et al.* 1996). Activated Rad53 and Mec1 phosphorylate specific targets, which can delay cell cycle transitions and induce DNA repair (Allen *et al.* 1994; Sidorova and Breeden 1997; Huang *et al.* 1998; Pellicioli *et al.* 1999; Sanchez *et al.* 1999; Bashkirov *et al.* 2000; Kihara *et al.* 2000). Rad53 is also important for monitoring DNA damage and nucleotide shortages within S phase (Paulovich and Hartwell 1995; Navas *et al.* 1996). Rad53 prevents firing of origins when DNA replication is encumbered by damage (Santocanale and Diffley 1998; Shirahige *et al.* 1998; Tersero and Diffley 2001) and potentially stabilizes stalled replication forks (Lopes *et al.* 2001). Rad53 also blocks premature metaphase-to-anaphase transition in cells with incompletely replicated DNA (Allen *et al.* 1994). Finally, Rad53 is an essential gene (Zheng *et al.* 1993) whose role in the unperturbed cell cycle is most likely involved in S-phase progression control (Desany *et al.* 1998; Dohrmann *et al.* 1999). Along with Mec1, Rad53 may act during S phase to remove the Sml1-mediated inhibition of ribonucleotide reductase (Zhao *et al.* 1998).

In this study, we demonstrate that Swi4-t overexpression can cause precocious entry into S phase in the absence of exogenous DNA damage. We also show that Swi4-t overexpression results in a loss of viability in combination with an allele of *RAD53* that is defective in checkpoint function (*rad53-11*). Analysis of S phase in *RAD* and *rad53-11* strains overexpressing Swi4-t suggests that Swi4-t prolongs the replicative state of cells and may increase the frequency of replication initiation. We propose that excessive origin firing can result in stalling of forks due to depletion of resources such as dNTPs or histones. In the absence of Rad53, stalled forks are not stable enough to resume replication when resources are replenished, and there is no signal to inhibit further origin firing. Combined, these deficiencies lead to lethality. In other words, during a normal S phase, Rad53 serves as a pacemaker, coordinating S-phase progression with growth conditions. By preventing excessive origin firing, it minimizes the effects of precocious S-phase entry imposed by hyperactive Swi4-t.

MATERIALS AND METHODS

Strains and plasmids: The yeast strain BY2006 *MAT***a** *ura3 leu2 trp1 his3* has been described (Sidorova and Breeden 1997). BY2390 is a *ura3* derivative of BY2007 *MAT***a** *ura3 leu2 trp1 his3 rad53-11::URA3* (Sidorova and Breeden 1997). BY2912 and BY2913 were derived from BY2006 and BY2390, respectively, by integration of *Escherichia coli* dam methylase on a pRS305 vector into the endogenous *LEU2* locus (Friedman *et al.* 1995). BY2887 and BY2888 are isogenic to BY2006 and BY2390, respectively, except they carry a *rad52::LEU2* disruption. BY2914 is *MAT***a** *ura3 lys2 leu2 trp1 pep4::HIS3 prb1Δ1.6R* with the endogenous *RAD53* gene tagged with the HA tag (Emili 1998). BY2226 *MAT***a** *ura3 leu2 trp1 his3 mec1-1::HIS3* and BY2227 *MAT***a** *ura3 leu2 trp1 his3 rad9Δ::LEU2* were described before (Sidorova and Breeden 1997). BY479 is *MAT***a** *dbf4-1 ura3 trp1 ade5*. Mating tester strains BY26 and BY27 are *MAT***a** *his1* and *MAT*α *his1*, respectively.

The plasmid pBD1168 is a YCp50 vector with *GAL::SWI4-t* and was described previously (Sidorova and Breeden 1993). pBD1411 and pBD1948 were described before (Tyers *et al.* 1993; Epstein and Cross 1994). The plasmid pBD2385 has been described previously (Sidorova and Breeden 1993) and is a Ycp50 with the *GAL* promoter-driven *SWI4* gene. The plasmid pBD2972 is a kind gift of Dr. S. Elledge and contains the *RNR1* open reading frame under the control of the *GAL* promoter on a 2-μm *TPR1* shuttle vector (Desany *et al.* 1998).

Growth conditions: All rich (YEP) and minimal (YC) media and growth conditions were as described before (Breeden and Mikesell 1991). Cultures used for elutriation were grown in YC-ura glucose media and then inoculated into YEP media with 2% raffinose and grown to OD_{660} = 1.5–1.8. To induce Swi4-t, galactose was added 80–100 min before the zero time point of the experiment (this induction time included the time in the elutriator). The zero time point was the time when the unbudded eluted cells were placed in the 30° incubator. This time was ample to fully induce Swi4-t overexpression, which takes ~30 min (J. Sidorova, unpublished results), and ensured that these cells had high levels of Swi4-t from the very beginning of G1 phase. For α-factor synchrony experiments, a culture at OD_{660} = 0.2 was typically arrested by incubation with 5 mg/liter of α-factor for 1 hr 45 min. Cells were released from the arrest by filtration or by addition of Pronase E (Sigma, St. Louis) to a final concentration of 10 mg/liter. *dbf4-1* strains were arrested at 37° for 2.5–3 hr and released into the cell cycle by shifting back to 25°.

FACS analysis: FACS analysis was done exactly as described before (Sidorova and Breeden 1997) except that cells were analyzed on a Calibur Analyser (Becton Dickinson, San Jose, CA).

Elutriation: Elutriation was performed in a J-6B centrifuge (Beckman, Fullerton, CA) using a JE-5.0 rotor and a 40-ml chamber (Beckman). The chamber was loaded with cells at 3500 rpm and 28 ml/min flow rate. Flow rate was then increased to 35 ml/min and the chamber was equilibrated with fresh media. Small G1 cell fractions were harvested in fresh media by further increasing the flow rate from 35 ml/min to 55 to 60 ml/min in 3- to 5-ml/min increments. The size of cells in these fractions was determined using a Z2 Particle Count and Size Analyzer and the data were analyzed using the AccuComp version 2.01 software (Beckman Coulter, Miami).

Nocodazole execution point measurements: To determine the nocodazole execution point, elutriated G1 cells were allowed to progress through the cell cycle. Aliquots (5 ml) of this culture were taken every 10 min, transferred to culture tubes with nocodazole (final concentration 12 mg/liter), and incubated at 30° for 2 hr on a roller drum. After 2 hr these cells were treated with 1% sodium azide and sonicated and the cell numbers were counted. As expected, cells treated with nocodazole before the first anaphase were arrested in the first cycle (Jacobs *et al.* 1988). Cells that had passed this transition divided once and arrested in metaphase of the second cycle. Thus, the final cell concentration after the nocodazole treatment was dependent upon the proportion of cells that had traversed the first anaphase and become nocodazole resistant at the time point when the aliquot was taken. Percentage of nocodazole-resistant cells at each time point was calculated using these cell concentration data. In addition, aliquots of cells were taken every 10 min from the untreated growing culture. These samples were also arrested by 1% sodium azide, and cell concentration and proportion of budded cells were determined in each sample.

***MAT* locus heterozygosity loss measurements:** These measurements were done essentially as described (Rose *et al.* 1990), taking advantage of the fact that diploid cells that lose one

of the *MAT* alleles will mate as haploids. *RAD/rad53-11* heterozygous diploids were constructed by mating BY2006 *MAT***a** *ura3 leu2 trp1 his3* (Sidorova and Breeden 1997) and BY2918 *MAT*α *ura3 leu2 his3 ade rad53-11::ura3::LEU2.* Diploids were transformed with the empty vector pYES2 or with the *GAL::SWI4-t* pBD1168 plasmid and were grown in YC-ura media with 2% raffinose and 2% galactose for about eight generations. Of these cells, 5×10^6 were mated with the equal number of cells of either one of the mating tester strains, BY26 or BY27. Upon plating, mated cells gave rise to prototrophic colonies. A separate control was included with only *RAD/rad53-11* and no mating tester strains. Neither *RAD/rad53-11* diploids nor mating tester strains alone gave rise to prototrophic colonies at a measurable frequency ($>10^{-6}$). Loss of heterozygosity was calculated as the number of mating events per milliliter of culture divided by the number of viable cells per milliliter of culture, and it was typically $\sim 10^{-4}$. The average numbers and average deviation of five measurements are reported.

Rad53 kinase assay: Immunoprecipitation of HA-Rad53 and kinase assays were done as described before (Sidorova and Breeden 1997) with 12CA5 mouse monoclonal antibodies to the HA epitope, except that no exogenous substrate was added to the kinase reaction.

Hemi-methylation analysis: Hemi-methylation analysis was done as described (Friedman *et al.* 1995, 1997). A constitutively expressed *E. coli* dam methylase gene on a pRS305 plasmid (Friedman *et al.* 1995) was integrated into the endogenous *LEU2* locus of yeast strains. These strains did not show a growth disadvantage compared to the parental strains, and *GAL::SWI4-t* overexpression had the same phenotype in the methylated strains as in the parental strains. DNA was digested overnight with *Dpn*I and *Eco*RI and loaded onto 0.8% agarose gels. Southern blotting and hybridization with the ARS607 probe were performed on GeneScreen membranes (New England Nuclear, Boston) according to the manufacturer's recommendations. The probe for the ARS607 region maps to chromosome VI coordinates from 202,454 to 203,480 (http://www-genome.stanford.edu/Saccharomyces/) downstream of the ARS607 ARS consensus sequence. In a hemi-methylated or unmethylated DNA, this probe preferentially hybridizes to a *Dpn*I-resistant *Eco*RI-digested fragment of ~3.0 kb ($DpnI^R$). Full methylation of DNA allows this fragment to be further digested by *Dpn*I. In this case, the probe hybridizes mostly to a *Dpn*I-sensitive ($DpnI^S$), *Eco*RI/*Dpn*I-digested fragment of ~1.0 kb.

Two-dimensional gel electrophoresis: We followed the previously described procedures for the DNA isolation and two-dimensional gel electrophoresis of replicative intermediates (Friedman and Brewer 1995). The isolated DNAs were digested with *Nhe*I for rARS replicative intermediates and resolved as follows. The first dimension separation was through 0.4% agarose at 1 V/cm for 20 hr at room temperature, and the second dimension was run in 1% agarose at 5 V/cm for 5 hr at 4°. To visualize ARS608, DNAs were digested with *Xho*I and resolved in 0.4% agarose at 1 V/cm for 24 hr in the first dimension and in 0.8% agarose at 2.7 V/cm for 15 hr at 4°. The probe for rARS maps to chromosome XII coordinates 460,050–460,777. The probe for ARS608 maps to chromosome VI coordinates 213,306–214,317.

RESULTS

Cells overproducing Swi4-t require a functional S-phase checkpoint for viability: We have shown before that the checkpoint-deficient allele of *RAD53* (*rad53-11*) or overproduction of a C-terminally truncated form of Swi4 (Swi4-t) both significantly reduce but do not completely eliminate the methyl methanesulfonate-dependent delay of the G1/S transition (Sidorova and Breeden 1997). To explore whether combining these two genetic determinants in one background will result in a complete elimination of this delay, we introduced three different *SWI4* alleles under control of the *GAL1* promoter into a *rad53-11* strain. These alleles encoded wild-type Swi4 and two of its truncated derivatives lacking 140 or 280 amino acids from the C terminus. We found that overexpression of the 280-amino-acid truncation Swi4-t from the *GAL* promoter (*GAL::SWI4-t*) severely abrogated the *rad53-11* strain's viability even in the absence of exogenous DNA damage (Figure 1A). These cells typically formed micro-colonies of up to 20 to 40 cells and division was arrested within 48 hr of growth on galactose.

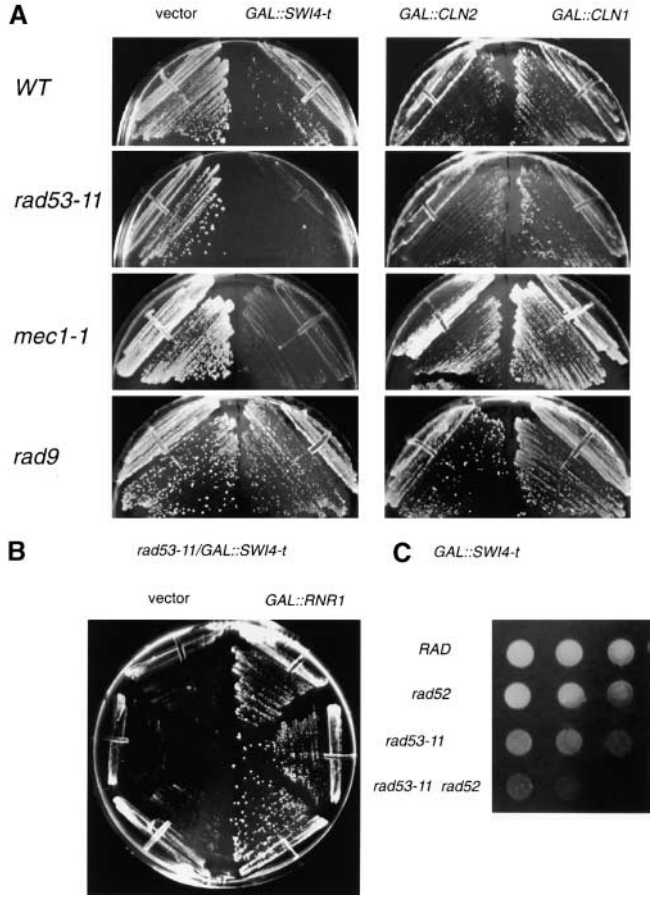

Figure 1.—Overexpression of Swi4-t is lethal in *rad53-11* or *mec1-1* mutants, but not in *rad9*Δ mutants. (A) Wild-type (BY2006), *rad53-11* (BY2390), *mec1-1* (BY2226), and *rad9*Δ (BY2224) strains were transformed with the empty vector (Ycp50), *GAL::SWI4-t* plasmid (pBD1168), *GAL::CLN1* plasmid (pBD1141), or *GAL::CLN2* plasmid (pBD1948) and streaked onto selective plates with galactose. (B) The *rad53-11* strain carrying *GAL::SWI4-t* was transformed with either the empty vector p414GAL1 or the *GAL::RNR1* plasmid (pBD2972). The strains were grown on selective media plates with galactose. (C) 10^5, 0.33×10^5, and 10^4 of cells of the wild-type (BY2006), *rad52*Δ (BY2887), *rad53-11* (BY2390), and *rad53-11 rad52*Δ (BY2888) strains carrying *GAL::SWI4-t* plasmid (pBD1168) were spotted onto selective media plates with galactose.

Neither the full-length Swi4 nor a shorter truncation of 140 amino acids compromised the strain's viability (data not shown).

Vallen and Cross (1995, 1999) have previously reported that high levels of either of the G1 cyclins, Cln1 or Cln2, are lethal in combination with the checkpoint-deficient *mec1* allele in the BF264-15D background. Since *CLN1* and *CLN2* levels increase upon induction of Swi4-t (Breeden and Mikesell 1994), we examined whether these cyclins were sufficient to cause lethality in the A364a *rad53-11* strain and whether *GAL::SWI4-t* was lethal to a *mec1* strain in the A364a background. Overexpression of Swi4-t did lead to poor viability with *mec1-1*. However, *GAL::CLN1* or *GAL::CLN2* did not abolish colony formation in *rad53-11* or *mec1-1* strains (Figure 1A). These results suggest that the lethality caused by Swi4-t in the A364a background cannot be attributed entirely to G1 cyclin overproduction.

Mec1 and Rad53 are required for all three DNA damage checkpoints in G1, S, and G2 phases of the cell cycle (Foiani *et al.* 2000). Rad9, on the contrary, is predominantly required for the G1 and G2 checkpoints (Navas *et al.* 1996). We found that Swi4-t overexpression had no effect on the viability of a *rad9*Δ strain (Figure 1A). The fact that Swi4-t is lethal in the *mec1-1* and *rad53-11* but not in the *rad9* background suggests that functions of Mec1 and Rad53 that do not overlap with Rad9's function are critical for protection against Swi4-t. Response to disruptions in DNA replication is one such function (Foiani *et al.* 2000).

If the lethal effect of Swi4-t on *rad53* cells is associated with the way the S phase progresses in these cells, we would anticipate that gene products important for proper S-phase progression might have synthetic phenotypes when overproduced in a *GAL::SWI4-t rad53-11* strain. *RNR1*, the large subunit of ribonucleotide reductase that is an important and perhaps limiting component of an S-phase cell, rescues the lethality of *GAL::SWI4-t rad53-11* cells when overexpressed (Figure 1B). Interestingly, *RNR1* is a target of Swi4 *in vivo* (Iyer *et al.* 2001) and *RNR1* is transcribed at a higher level when Swi4 or Swi4-t is overexpressed (data not shown).

The presence of Swi4-t could generate some kind of damage during replication, which could evoke the Rad53-dependent checkpoint and necessitate repair. Indeed, *RAD52*, which is critical for DNA recombination and repair (Sung *et al.* 2000), has a synthetic phenotype with *rad53-11 GAL::SWI4-t*. The *rad52* deletion exacerbates Swi4-t-induced lethality in *rad53-11* cells while having no measurable effect on *RAD53* cells (Figure 1C). In addition, long-term Swi4-t overexpression results in an increase in chromosome instability as judged by the loss of heterozygosity of the *MAT* locus. A *RAD/rad53-11* heterozygous diploid strain overexpressing Swi4-t shows about a threefold higher frequency of allele loss events at the *MAT* locus than cells carrying the vector alone ($1.25 \pm 0.33 \times 10^{-4}$ *vs.* $4.18 \pm 0.66 \times 10^{-4}$).

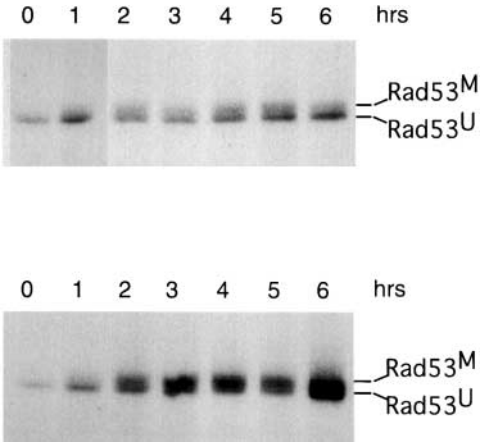

Figure 2.—*GAL::SWI4-t* induces Rad53 kinase activity. A wild-type strain (BY2914) with a HA-tagged Rad53 was transformed with the empty vector pYES2 (top) or with the *GAL::SWI4-t* plasmid (pBD1168, bottom). The cells were grown in selective media with raffinose and then diluted into fresh media with galactose to induce Swi4-t expression. Aliquots were taken immediately before galactose was added and every hour for 6 hr. Rad53 was immunoprecipitated with HA antibodies and allowed to autophosphorylate in the presence of [γ-$^{32}PO_4$] ATP. Products of the kinase reactions were resolved on an SDS polyacrylamide gel and autoradiographed. Positions of the modified and unmodified forms of Rad53 are marked by lines on the right.

Swi4-t induces Rad53 kinase activity and speeds up the G1/S transition: DNA damage or stalled replication forks cause modification and activation of the Rad53 kinase. This modification results in the appearance of a low-mobility form of Rad53 on protein gels (Allen *et al.* 1994; Sanchez *et al.* 1996; Sun *et al.* 1996). Figure 2 shows the state of Rad53 immunoprecipitated out of wild-type cells harboring vector control plasmid or *GAL::SWI4-t* and grown in galactose. An unmodified, high-mobility form of Rad53 remains the predominant form of this kinase in vector control cells throughout the time course. In contrast, after 2 hr of galactose induction of Swi4-t, there is a detectable accumulation of the low-mobility form of Rad53. Since typically only the unmodified form of Rad53 is readily detectable during a normal cell cycle (Sun *et al.* 1996; J. Sidorova, data not shown), this increased presence of the low-mobility, modified form of Rad53 upon induction of Swi4-t suggests that these cells are undergoing a Rad53-mediated checkpoint response.

Swi4 is a critical activator of the G1/S transition, and Swi4-t is a hyperactive form that deregulates transcription of G1 cyclins (Breeden and Mikesell 1994). As such, Swi4-t overproduction is expected to affect the G-to-S transition. However, it is less evident that Swi4-t may affect S-phase progression. We used centrifugal elutriation to examine cell cycle progression in Swi4-t cells with or without functional Rad53. Swi4-t was induced 120 min before the first measurement was taken. This protocol enabled us to look at the immediate effects of

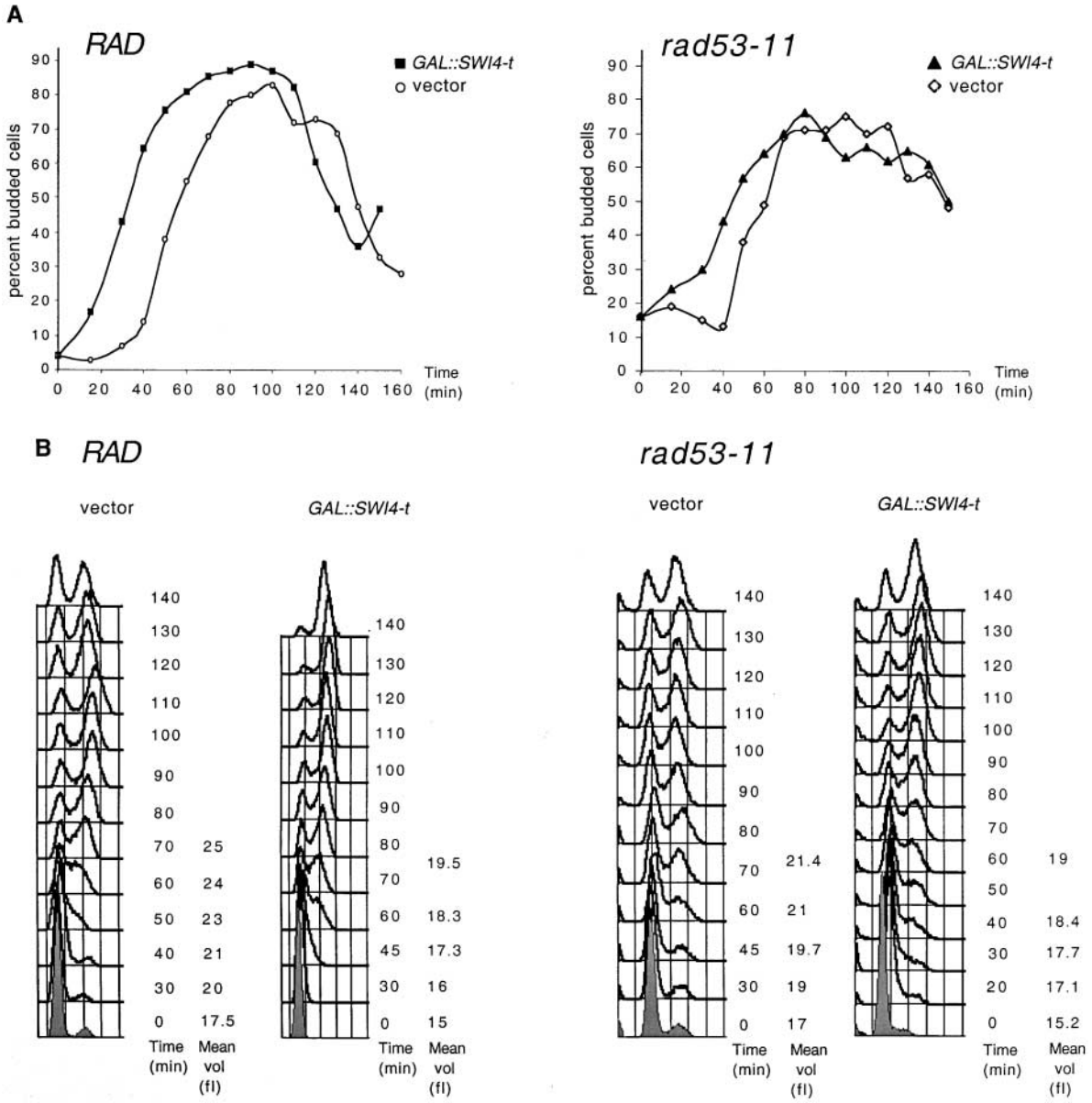

FIGURE 3.—*GAL::SWI4-t* speeds up budding and S-phase onset in elutriated G1 cells. (A) Wild-type (BY2912) or *rad53-11* (BY2913) cells were transformed with *GAL::SWI4-t* (pBD1168) or empty vector (Ycp50) and grown in rich medium with raffinose, galactose was added for 20–40 min, and small G1 cells were harvested by centrifugal elutriation in fresh YEP media with raffinose and galactose. Fractions of identical cell size distribution were collected and allowed to progress through the cell cycle. Samples were taken from these cultures every 10–15 min and the percentage of budding cells was determined. (B) Wild-type strain fractions and *rad53-11* strain fractions obtained by elutriation were subjected to FACS analysis. Mean cell volumes at the corresponding time points are indicated to the right of the profiles.

GAL::SWI4-t on the cell cycle. The fractions collected from the elutriator were allowed to progress through the cell cycle and sampled at 10-min intervals. Overexpression of Swi4-t did not change the kinetics of cell growth in G1 (data not shown), but it caused cells to bud at a smaller size than controls. For example, *GAL:SWI4-t* cells achieved 50% budding at the average mean volume of 17.4 fl, while the vector control cells were 50% budded at the average mean volume of 24 fl. Direct comparison of fractions of cells with identical starting size distributions showed that there was a 25-min difference in the initiation of budding of the control *vs.* Swi4-t cells (Figure 3A). As with control cells, the budding induced by Swi4-t was accompanied by an earlier entry into S phase as determined by FACS (Figure 3B), indicating that these events remain coupled in the Swi4-t cells. *rad53-11* cells carrying Swi4-t reproducibly exited G1 at a smaller size than control cells. However, the volume difference between *GAL::SWI4-t* and vector cells was less dramatic than in the case of the wild-type cells (20 fl *vs.* 23 fl; see also Figure 3, A and B). We also found that elutriated *rad53-11* cells exhibited a less synchronous exit from G1

TABLE 1

Timing of the G1-to-S and metaphase-to-anaphase transitions

Strain	Starting mean volume (fl)	50% budding (G1/S transition, min)	50% nocodazole resistance (metaphase-to-anaphase transition, min)	Time between the transitions
RAD/vector	15.62	57	134	77
RAD/GAL::SWI4-t	15.67	34	120	86
rad53/vector	16.84	62	143	81
rad53/GAL::SWI4-t	16.72	46	>200[a]	>154

Cells of the genotypes indicated were grown in raffinose and then galactose was added 20 min prior to the elutriation. G1 cells of the indicated size were harvested and their passage through the cell cycle was followed. Cell number and percentage of budded cells were determined at each time point. After 40 min, aliquots of each culture were removed every 10 min and incubated with 12 mg/liter of nocodazole for 2 hr. These cells were counted and the percentage of cells that had passed the nocodazole execution point and were able to divide once was calculated.

[a] *rad53-11 GAL::SWI4-t* cells did not exceed 50% nocodazole resistance within the time frame of the experiment.

than their wild-type counterparts. Budding was slower and 1N DNA persisted for a longer time in these cells (Figure 3B).

Comparison of budding indices of the synchronized *GAL::SWI4-t* and vector cells indicates that *GAL::SWI4-t* cells may spend more time as budded cells (Figure 3A). Moreover, the FACS data (Figure 3B) suggest that while *GAL::SWI4-t* cells traverse the G1-to-S transition earlier than controls, they spend a longer time completing S phase. In *GAL::SWI4-t* cells, the fraction of cells with 2N DNA content gradually rises between 60 and 140 min with no indication of progression into the next cell cycle. In contrast, the control cells show no pausing at the 2N or near 2N DNA stage and proceed rapidly into the next cycle. To address this in more detail, we obtained elutriated populations of cells with identical starting size and monitored their progression between G1 and G2 phases. Swi4-t cells budded 23 min earlier than controls (Table 1). However, the execution of anaphase, as measured by the acquisition of 50% resistance to the microtubule-destabilizing drug nocodazole, occurred only 14 min earlier in Swi4-t cells compared to controls (Table 1). Hence, it appears that Swi4-t cells spend more time between the end of G1 phase and anaphase. *rad53-11* cells overexpressing Swi4-t also experienced an extension of this interval (Table 1). *rad53-11 GAL::SWI4-t* cells were less efficient in completing the cell cycle, since the number of nocodazole-resistant cells never exceeded 50%. This suggests that these *GAL::SWI4-t* cells do not undergo premature mitosis due to mutation in *RAD53*.

Extended replicative state in cells expressing Swi4-t: The extension of the interval between G1 and anaphase in cells overexpressing Swi4-t suggests that Swi4-t may slow or impair DNA replication. To follow the replication more directly, we employed hemi-methylation analysis (Friedman *et al.* 1997). We used the strains that constitutively express *E. coli* dam methylase, which methylates both strands of yeast DNA *in vivo*. In strains carrying dam methylase, replication through a particular region can be followed as a transient state of hemi-methylation, because hemi-methylated DNA is resistant to cleavage by the *Dpn*I restriction enzyme. To compare *GAL::SWI4-t* and vector cells, elutriated G1 cells of the two strains were followed as they progress through G1 and S, but were arrested in anaphase by adding microtubule inhibitor nocodazole (Figure 4A). Alternatively, we employed α-factor synchronization (Figure 4, B–D). This method induces a higher degree of synchrony but the first G1-to-S transition is very rapid.

Consistent with previous observations, in elutriated wild-type G1 cells arrested before anaphase by nocodazole, the onset of S phase and replication through the ARS607 region as assayed by *Dpn*I resistance occurred earlier in *GAL::SWI4-t* cells than in controls and just as efficiently, as indicated by the sharp increase of hemi-methylation level (Figure 4A). However, despite the earlier onset of replication, the hemi-methylated state of the ARS607 locus persisted at a high level for a longer time in *GAL::SWI4-t* cells than in controls.

Using the α-factor synchronization we observed a rapid G1-to-S transition in both *GAL::SWI4-t* and vector cells (Figure 4B). This was expected because α-factor-arrested cells exceed the critical size needed for the transition into S phase and thus exit G1 very quickly upon release. Nonetheless, the hemi-methylation assay showed that while both *GAL::SWI4-t* and vector control cells started to accumulate newly replicated, hemi-methylated DNA in the early ARS607 locus at about the same time, *GAL::SWI4-t* cells carried hemi-methylated DNA for a longer time than vector controls (Figure 4, C and D). In *rad53-11* cells, induction of hemi-methylation was low due to a low basal level of methylation, precluding a definitive interpretation of results.

The hemi-methylation studies suggest that Swi4-t overexpression can result in both an earlier appearance and a prolonged presence of nascent DNA in the ARS607 region in wild-type cells. However, it is also possible that the observed differences in hemi-methylation profiles are caused by an altered accessibility to dam methylase

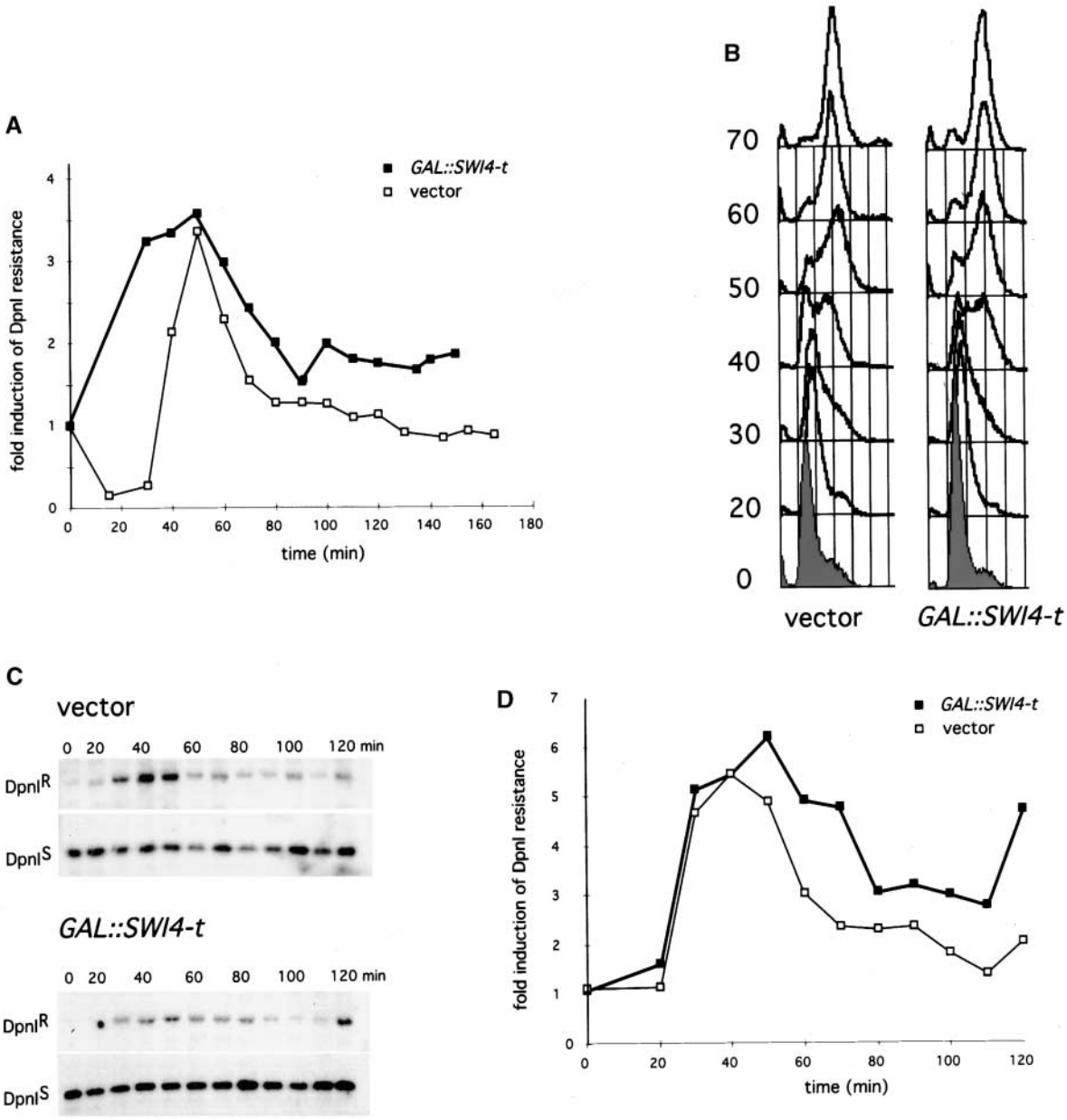

FIGURE 4.—*GAL::SWI4-t* extends the interval during which nascent DNA can be detected by hemi-methylation analysis. (A) The wild-type (BY2912) strain transformed with the empty vector (Ycp50) or with *GAL::SWI4-t* (pBD1168) was elutriated as described for Figure 3. Mean volumes of the starting cultures were 16.4 fl for both wild-type strains. These cultures were allowed to progress into S phase, and after 40 min nocodazole was added to the final concentration of 12 mg/liter to prevent escape into the second cell cycle. Samples were taken every 10 min to isolate DNA and methylation state of nascent DNA was followed by digesting with *Dpn*I and *Eco*RI. The Southern blots of *Eco*RI, *Dpn*I-digested DNA were hybridized with probes to the ARS607 region and quantified. The *Dpn*I resistance was determined as a ratio of the *Dpn*I-resistant (*Dpn*I^R) to the sum of the *Dpn*I-resistant and *Dpn*I-sensitive (*Dpn*I^S) DNA. The *Dpn*I resistance at time point 0 was set equal to 1 unit, so that the fold induction of *Dpn*I resistance could be compared for the indicated strains. (B–D) The wild-type (BY2912) strain transformed with the empty vector (pBD1129) or with *GAL::SWI4-t* (pBD1168) was synchronized in late G1 by incubating with α-factor for 105 min. Galactose was added 30 min before the release. Cells were released from the arrest by filtration and resuspended in fresh media with raffinose and galactose. Aliquots of cultures were taken at 10-min intervals and analyzed by FACS (B) and hemi-methylation analysis (C). Southern blot hybridizations to ARS607 region DNA are shown (C), and quantitation of these data is shown in (D). Hemi-methylated DNA is resistant to *Dpn*I digest, giving rise to a large *Eco*RI-*Eco*RI fragment (*Dpn*I^R), and fully methylated DNA is sensitive to *Dpn*I digest, giving rise to a smaller *Eco*RI-*Dpn*I-digested (*Dpn*I^S) fragment.

after one round of replication, for example, due to a change in the nascent chromatin structure.

Swi4-t overexpressing cells display more replicative intermediates during S phase: To test whether extension of the hemi-methylated state in DNA upon Swi4-t overexpression correlates with the prolonged replicative state and to assess replication in *rad53-11* cells, we employed two-dimensional (2D) gel electrophoresis (Figure 5A;

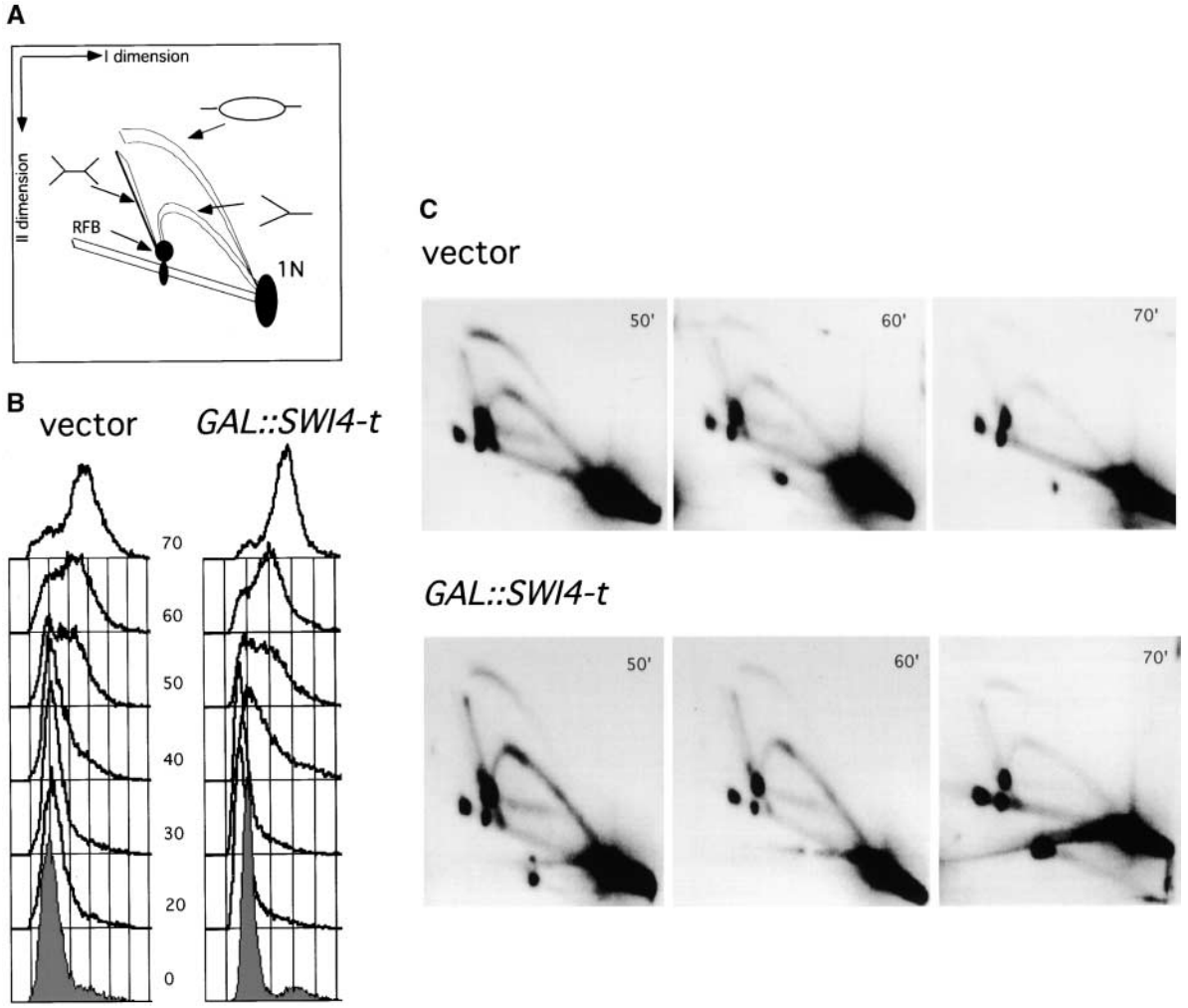

FIGURE 5.—*GAL::SWI4-t* extends the window of activity of the ribosomal ARS in wild-type cells. (A) Diagram depicting the migration patterns of replication intermediates of ribosomal DNA through 2D gels. (B) A wild-type (BY2006) strain transformed with the empty vector (Ycp50) or with *GAL::SWI4-t* (pBD1168) was synchronized with α-factor. Galactose was added 30 min before the release. Cells were released from the arrest by adding Pronase E (Sigma) to the final concentration of 10 mg/liter and aliquots of cultures were taken at specified times for FACS analysis (B) and 2D gel analysis (C). The DNAs were digested with the *Nhe*I restriction enzyme and resolved in two dimensions. Gels were subjected to Southern blotting and hybridization and probed for the ribosomal ARS locus as specified in MATERIALS AND METHODS.

FRIEDMAN and BREWER 1995). In these gels, bubble-shaped intermediates arise from origin firing within the given DNA fragment. Y-shaped intermediates are generated either when forks are initiated distally and passively replicate through the monitored fragment or when the replication bubble is positioned asymmetrically within the fragment and one fork completes its replication before the other. To quantify replicative intermediates, signal intensities from bubbles or Ys can be normalized to that of the double-stranded DNA, which migrates as a spot in front of the replicating molecules (1N DNA, Figure 5A; IVESSA *et al.* 2000).

Using this assay, we first followed replication through the ribosomal gene cluster on chromosome XII, which spans 1000–2000 kb of 9-kb repeats of rDNA each containing an ARS sequence (rARS). Replication intermediates of the rDNA cluster are detectable throughout S phase (Figure 5C). In addition to bubble- and Y-shaped molecules, two other types of replicative intermediates can be detected in rDNA during replication. Leftward-moving forks that stall at the *r*eplication *f*ork *b*arrier (RFB) show up as a dot of increased intensity on the Y arc (LINSKENS and HUBERMAN 1988; BREWER *et al.* 1992). In addition, at later stages of replication, converging forks form X-shaped, Holliday junction-like intermediates, which migrate separately from simple Ys (Figure 5, A and C). Thus, the rDNA cluster provides an opportunity to monitor accumulation of the regular early (bubble) and late (Y) intermediates, including the stalled and converged replicative forks.

To follow replication, cells were synchronized in late G1 by α-factor. The highest levels of replicative intermediates in the wild-type cells were detectable at 50 min (Figure 5, B and C). At this time and later, we could

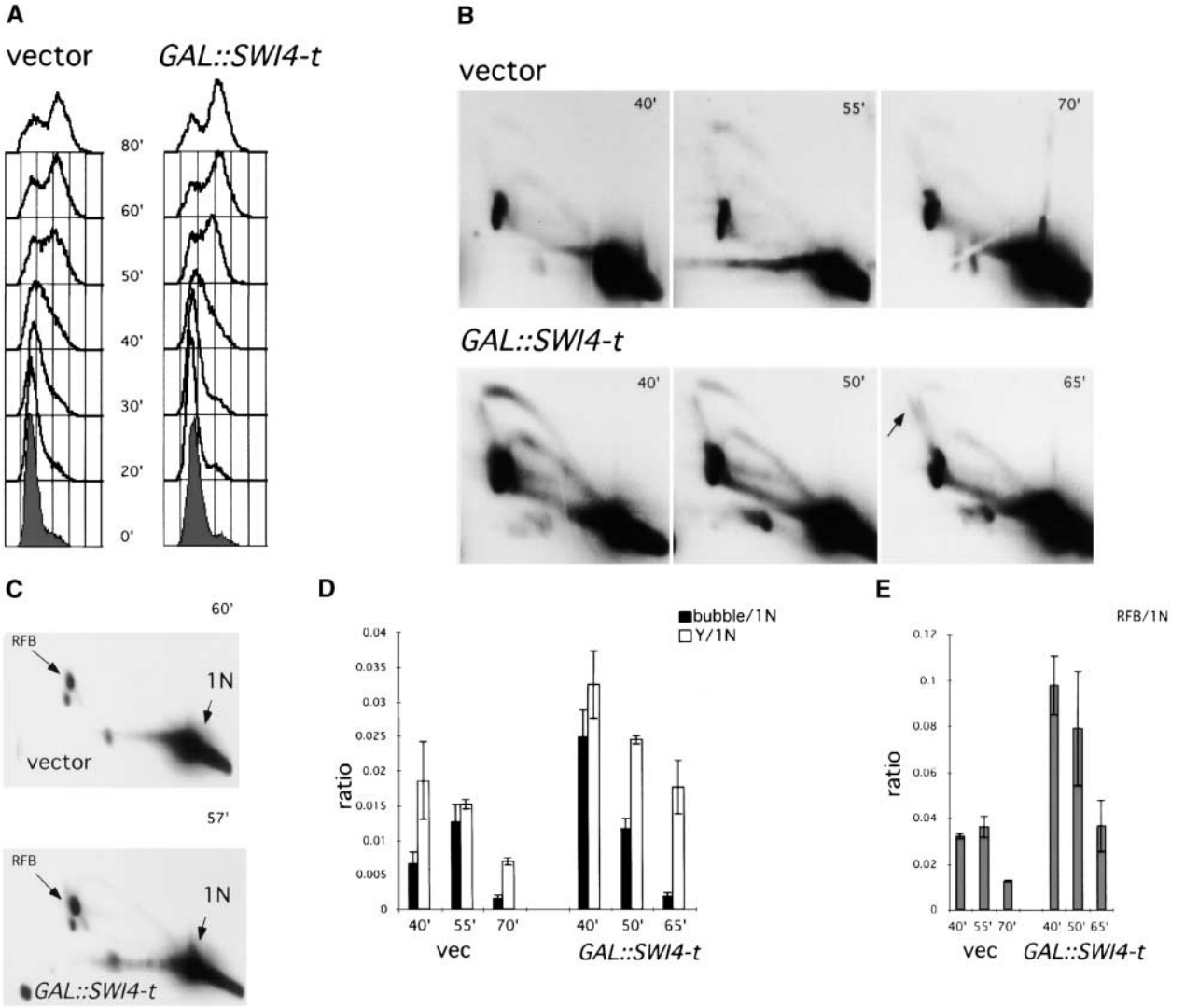

FIGURE 6.—*GAL::SWI4-t* increases the levels of replicative intermediates detectable in *rad53-11* cells. A *rad53-11* (BY2390) strain transformed with the empty vector (Ycp50) or with *GAL::SWI4-t* (pBD1168) was treated as described for the wild-type strain in the legend to Figure 5. Samples of cells were taken every 10 min to determine DNA content by FACS. (A) Note that *GAL::SWI4-t* cells are slightly ahead of the vector controls in S-phase progression. (B) DNAs isolated from these cultures at the times specified were digested with the *Nhe*I restriction enzyme and resolved in two dimensions as in Figure 5. The arrow points to the X-shaped intermediates prominent in the *rad53-11 GAL::SWI4-t* cells. (C) A shorter exposure of the two-dimensional gel from an independent experiment is shown to compare the abundance of the RFB-stalled forks in the *rad53-11* vector *vs. rad53-11 GAL::SWI4-t* cells. Positions of RFB-stalled forks and of 1N DNA are marked by arrows. (D and E) Autoradiograms of rARS replicative intermediates of the *rad53-11* BY2390 strain with the empty vector or *GAL::SWI4-t* were quantified, and the signals in bubbles and Ys (D) or in the RFB-stalled fork (E) intermediates were normalized to the signal in 1N DNA and plotted. The average of two measurements is presented.

detect higher levels of Y intermediates in *GAL::SWI4-t* cells compared to controls, suggesting that replicative intermediates persist for a longer time in these cells. In *rad53-11* cells, the effect of Swi4-t overexpression was more pronounced (Figure 6). The *rad53-11 GAL::SWI4-t* cells maintained higher levels of bubbles, Ys, and stalled and converged forks than did the *rad53-11* vector controls (Figure 6B). Figure 6C shows a repeat of this experiment with a lighter exposure so that the levels of the RFB-stalled forks in *rad53-11 GAL::SWI4-t vs. rad53-11* vector cells can be compared (marked by arrows). Quantitation of these gels confirms the observation that the presence of *GAL::SWI4-t* results in about twice as many Ys and RFB-stalled forks (Figure 6, D and E). Thus, the two-dimensional gel analysis suggests that replicative forks are present on rDNA for an extended time in *GAL::SWI4-t* cells. In addition, the levels of bubble intermediates evident in a fixed amount of DNA are two- to threefold higher in *rad53-11 GAL::SWI4-t* cells than in controls at the earliest time point (see Figure 6, B and D). These data suggest that the ribosomal ARS fires more frequently when *GAL::SWI4-t* is present.

***rad53-11* and *GAL::SWI4-t* may influence origin firing frequency:** We next followed replication of a single copy origin, ARS608, whose firing frequency is variable and limited to a narrow window of time in the first half of S phase (Friedman *et al.* 1997; Yamashita *et al.* 1997). As seen in Figure 7A, both in *RAD* and in *rad53-11* cells, *GAL::SWI4-t* extended the window of time during which the large bubble intermediates (marked by arrows) were detectable in ARS608 DNA by at least 10 min. Y intermediates in *GAL::SWI4-t* cells were also more prevalent late in S phase.

Interestingly, we also observed a difference between the relative amounts of Y and bubble intermediates in *RAD* and *rad53-11* cells. An important difference between the rARS and ARS608 is the fact that the latter is a single-copy ARS and it is replicated either actively, if it fires, or passively from the nearby ARS607. Thus, the bubble-to-Y ratio is a good indicator of the firing efficiency of ARS608 (Friedman *et al.* 1997; Yamashita *et al.* 1997). As Figure 7B shows, the maximal detectable firing frequency of ARS608 was reproducibly increased about twofold in *rad53-11* cells. The quantification also showed that in both *RAD* and *rad53-11* cells, Swi4-t overexpression did not lead to a significant increase in the ARS608 bubble-to-Y ratio during the peak activity of this ARS. Overall, the 2D analysis indicates that both *rad53-11* and *GAL::SWI4-t* contribute to the increase in origin firing although these contributions are manifested differently. While *rad53-11* shifts the distribution toward early intermediates, *GAL::SWI4-t* extends the window of time during which the intermediates are detectable.

To further address the effects of Swi4-t, we asked whether it can suppress replication initiation mutations. *DBF4* is an essential gene required for initiation but not elongation of replication throughout S phase (Johnston and Thomas 1982; Bousset and Diffley 1998; Donaldson *et al.* 1998). *dbf4-1* is a temperature-sensitive mutation, which reduces the Dbf4/Cdc7 complex kinase activity (Kihara *et al.* 2000) and prevents replication initiation at the nonpermissive temperature (Johnston and Thomas 1982). We found that Swi4-t, but not the full-length Swi4, improved the growth of the *dbf4-1* strain at the semi-permissive temperature of 33° (Figure 8A). We then arrested the *GAL::SWI4-t* and vector cells at the beginning of S phase by shifting them to 37°, induced Swi4-t expression, and then shifted them back to the permissive temperature. FACS analysis showed that the *dbf4-1*

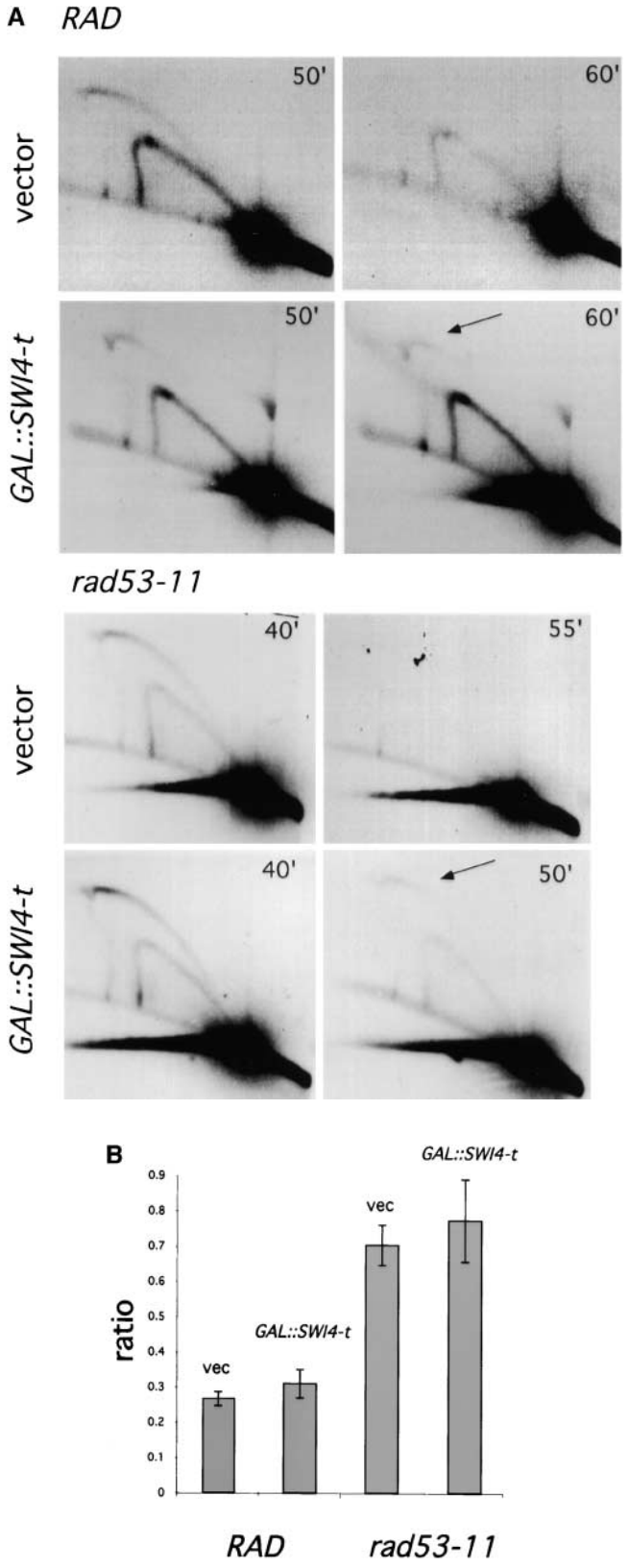

Figure 7.—*GAL::SWI4-t* extends the window of time during which ARS608 replicates. (A) The wild-type (BY2006) or *rad53-11* (BY2390) strains with the empty vector or *GAL::SWI4-t* presented in Figures 5 and 6 were used to examine ARS608 replication. DNAs from specified time points were digested with the *Xho*I restriction enzyme and resolved in two dimensions as specified in materials and methods and probed with ARS608 locus DNA. (B) Three to four blots of ARS608 replicative intermediates, taken from the times when ARS608 showed the most activity, were quantified using PhosphoImager for each strain and the ratio of the signals in the full arc of bubble intermediates and the full arc of Y intermediates was averaged and plotted.

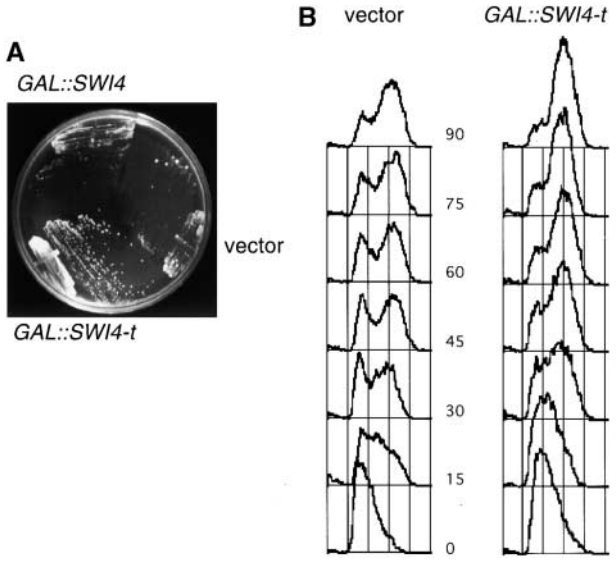

FIGURE 8.—*GAL::SWI4-t* partially suppresses temperature sensitivity of the *dbf4-1* strain. (A) A *dbf4-1* (BY479) strain transformed with the empty vector (Ycp50), *GAL::SWI4* (pBD2385), or *GAL::SWI4-t* (pBD1168) was streaked onto selective media plates with galactose and grown at 33°. (B) The *dbf4-1* (BY479) strain with the empty vector (Ycp50) or with *GAL::SWI4-t* (pBD1168) was grown at 25° in YEP media with raffinose and arrested before S phase by incubation at 37° for 2.5 hr. Galactose was added 30 min prior to the release. The cultures were released from the arrest by shifting back to 25°. Aliquots of these cultures were taken every 15 min and subjected to FACS analysis.

cells that overexpressed Swi4-t completed S phase significantly faster than controls, suggesting that Swi4-t may increase initiation in *dbf4-1* cells (Figure 8B).

DISCUSSION

Swi4-t is a hyperactive derivative of the late G1-specific transcriptional activator Swi4. When overexpressed, it causes Swi4 target genes to be ectopically expressed at all stages of the cell cycle. In this study, we demonstrated that it can cause precocious entry into S phase. In addition, we observed that even though *GAL::SWI4-t* cells can enter S phase earlier than normal cells, they spend more time between the end of G1 and anaphase. Hemimethylation analysis suggests that Swi4-t overexpression leads to an extension of the time during which nascent DNA is generated in a given region. If that is the case, it could result from DNA reduplication or from gap filling or strand break repair, or any combination thereof. 2D gel analysis specifically suggests that Swi4-t overexpression correlates with a prolonged presence and/or increased abundance of replicative intermediates. A higher level of replicative forks could be detected in rDNA in *GAL::SWI4-t* cells as compared to vector cells. Also, bubble and Y intermediates were detectable for a longer time in ARS608 DNA in *GAL::SWI4-t* cells.

The fact that we could detect an extended window of time during which bubbles and Ys were present in rARS and ARS608 DNA in cells carrying *GAL::SWI4-t* suggests that some replicative forks in these regions were slowing or stalling. Another interpretation of this result is that Swi4-t may cause an extension of the window of time within which origins fire and/or an increase in the frequency with which they fire. Support for the notion that Swi4-t may increase efficiency of at least some origins is rendered by the fact that Swi4-t overexpression partially suppresses the temperature sensitivity of the *dbf4-1* strain and speeds up the course of S phase in *dbf4-1* cells. Dbf4 is involved in initiation of DNA replication (JOHNSTON and THOMAS 1982), and one may expect that the consequences of partial Dbf4 inactivation could be counteracted by upregulating origin firing.

The cellular response to Swi4-t involves Rad53. Rad53 is required for viability, and when hyperactivated in response to DNA damage, it slows down S-phase progression by inhibiting origin firing (SANTOCANALE and DIFFLEY 1998; SHIRAHIGE *et al.* 1998; TERSERO and DIFFLEY 2001). Swi4-t overexpression leads to the activation of the Rad53 kinase. *rad53-11* checkpoint mutant cells are not able to tolerate overexpression of Swi4-t and lose viability within three to five divisions. These observations suggest that Swi4-t overexpression invokes a checkpoint response. Our data indicate that *rad53-11 GAL::SWI4-t* cells do not enter into or progress through S phase any faster than *RAD GAL::SWI4-t* cells. However, *rad53-11* in combination with Swi4-t results in a further increase in the levels of both early (bubble) and late (stalled and converged forks) rDNA replicative intermediates compared to controls. *rad53-11* also results in a significant increase in the firing frequency of ARS608. This latter observation is consistent with the previous report by SHIRAHIGE *et al.* (1998) made with a *rad53-1* allele of *RAD53*, but differs from the study of ARS301 in a *rad53-21* strain where no increased firing was found (SANTOCANALE *et al.* 1999). This discrepancy is probably due to differences between these *RAD53* alleles. Only a subset of *RAD53* mutations suppresses the temperature-sensitive initiation defect of *dbf4-1*, and *rad53-21* is synthetically lethal with *dbf4-1* at the permissive temperature (DESANY *et al.* 1998; DOHRMANN *et al.* 1999; KIHARA *et al.* 2000).

We propose a simple interpretation of the data presented in this study, which is that both *GAL::SWI4-t* and *rad53-11* affect initiation of replication, albeit to a different extent. In a normal cell, Swi4 activity is rate limiting for S-phase entry (MCINERNY *et al.* 1997). Elevating Swi4 activity with a stable, constitutively produced form of Swi4 may not only erroneously signal the cell to enter S, but may also signal its capacity to replicate at a high rate. This could lead to an increase in the number of replicative forks concurrently present on DNA. When the density of forks is elevated above what a given growth condition can support, this puts strain on the cell. Elevated fork density could deplete dNTP and/or histone pools,

increase torsional stress on DNA, or make other key components of the replication machinery rate limiting. The consequences of these adversities may be transient stalling or slowing of fork movement.

Rad53 plays a critical role in mitigating the effects of Swi4-t on replication. In a *RAD* cell, forks that stall or slow down may recruit Rad53. By stabilizing these forks with the replicative machinery assembled on them, Rad53 allows most of them to resume replication when supplies are replenished (Desany *et al.* 1998; Lopes *et al.* 2001). Stalled forks "marked" with activated Rad53 send a signal inhibiting further origin firing and thus prevent further accumulation of replicative intermediates and the unproductive spending of origins. Thus, the excessive firing promoted by *GAL::SWI4-t* is neutralized. In *rad53-11* cells, this protective mechanism is deactivated, leading to the accumulation of many unstable replicative intermediates, which may require disassembly and processing through a recombination pathway to complete replication (Rothstein *et al.* 2000). In agreement with this, the defect in recombination caused by a *rad52* disruption further exacerbates Swi4-t-induced lethality in *rad53-11* cells, but has no detectable effect on *RAD53* Swi4-t cells.

There is growing evidence that abundance of stalled forks during replication may contribute to genome destabilization. For example, mutations in the rDNA-specific helicase, Pif1, decrease the number of stalled forks, and this is correlated with a decrease in the amount of rDNA breakage and a reduction in the number of rDNA circles (Ivessa *et al.* 2000). Our work suggests the possibility that cells that experience precocious S phase due to a hyperactivation of a G1-to-S transition regulator acquire genomic instability precisely because these regulators promote an increase in the number of stalled replicative forks.

Finally, a number of hypotheses can be entertained to explain the mechanism by which hyperactive Swi4-t signals S-phase entry. Similar to other transcription factors, a DNA-bound Swi4-t could promote initiation of replication *in cis* (Van de Vliet 1999). An interesting possibility is raised by the recent report by Maser *et al.* (2001), which finds enhanced binding of mammalian E2F1 near active replication origins. This and other findings (Royzman *et al.* 1999) imply that transcriptional activators important for the G1-to-S transition could have a direct role in DNA replication control.

It is also possible that the effect of Swi4-t on S-phase progression is mediated by the altered expression of Swi4 target genes. Two critical targets of the Swi4 activator, which are rate limiting for the G1-to-S transition, are the G1 cylins *CLN1* and *CLN2*. Overproduction of *CLN1* or *CLN2* is lethal in combination with *mec1* and, to a lesser degree, *rad53* mutations in some backgrounds (Vallen and Cross 1995, 1999). However, *CLN* overproduction is not sufficient to kill either *mec1-1* or *rad53-11* cells in the A364a background. This difference may be attributed to the state of the *SML1* locus (Zhao *et al.* 1998); however, this locus is certainly not the only one that differs between these strains. In *mec1* and *rad53* cells, Sml1-mediated inhibition of the ribonucleotide reductase is not relieved (Zhao *et al.* 1998) and thus dNTP levels may be limiting. In the Vallen and Cross study (1999), the lethality of G1 cyclin overexpression to the *mec1* mutants could be explained by a concomitant decline in the mRNA level of the large subunit of the ribonucleotide reductase Rnr1 (Vallen and Cross 1999), which further depletes dNTP pools. In our case, however, Swi4-t overproduction is lethal to *rad53-11* cells even though it upregulates *RNR1* transcription (J. Sidorova, unpublished data), and *SML1* is inactivated (Paulovich *et al.* 1997). Hence, Rnr1 is not likely to be the cause of the Swi4-t-induced lethality, even though it can mitigate the lethal consequences of Swi4-t overproduction, when it, too, is overexpressed. Moreover, suppression of the lethality of *GAL::SWI4-t* can also be achieved by overexpression of *SRL1* (J. Sidorova, unpublished data), and thus it is not specific to *RNR1*. *SRL1*, whose function is unknown, is another Swi4-activated gene (Iyer *et al.* 2001), and like *RNR1*, it can suppress the lethality of the *rad53* deletion when highly overexpressed (Desany *et al.* 1998). All these observations suggest that the Swi4-t-induced lethality cannot be tracked to one Swi4-t target gene and may instead result from altered expression of many targets.

A dividing eukaryotic cell faces intrinsic challenges that arise from its ability to initiate DNA replication from many loci throughout the genome. It seems that for an efficient S phase, it may be critically important to adjust origin-firing efficiency depending on the cellular resources as well as to have a negative feedback constantly monitoring the progress of replication.

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