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Novel Role for Checkpoint Rad53 Protein Kinase in the Initiation of Chromosomal DNA Replication in *Saccharomyces cerevisiae*

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ABSTRACT

A novel role for Rad53 in the initiation of DNA replication that is independent of checkpoint or deoxynucleotide regulation is proposed. Rad53 kinase is part of a signal transduction pathway involved in the DNA damage and replication checkpoints, while Cdc7-Dbf4 kinase (DDK) is important for the initiation of DNA replication. In addition to the known *cdc7-rad53* synthetic lethality, *rad53* mutations suppress *mcm5-bob1*, a mutation in the replicative MCM helicase that bypasses DDK's essential role. Rad53 kinase activity but neither checkpoint FHA domain is required. Conversely, Rad53 kinase can be activated without DDK. Rad53's role in replication is independent of both DNA and mitotic checkpoints because mutations in other checkpoint genes that act upstream or downstream of *RAD53* or in the mitotic checkpoint do not exhibit these phenotypes. Because Rad53 binds an origin of replication mainly through its kinase domain and *rad53* null mutants display a minichromosome loss phenotype, Rad53 is important in the initiation of DNA replication, as are DDK and Mcm2–7 proteins. This unique requirement for Rad53 can be suppressed by the deletion of the major histone H3/H4 gene pair, indicating that Rad53 may be regulating initiation by controlling histone protein levels and/or by affecting origin chromatin structure.

ELABORATE regulatory mechanisms have evolved in eukaryotic cells to ensure that DNA replication occurs only once per cell cycle (for recent reviews see Bell and Dutta 2002; Diffley 2004). First, a multiprotein prereplication complex (pre-RC) is assembled onto origins of replication during the G1 phase of the cell cycle. Origins are bound throughout the cell cycle by a six-member protein complex known as the origin recognition complex (ORC). Cdc6, which is produced in the G1 phase, together with Cdt1 protein loads the multimeric minichromosome maintenance (MCM) complex onto the ORC. The pre-RC is then activated by two independent protein kinases: cyclin-dependent kinase (CDK) (Cdk1-Clb) and Dbf4-dependent kinase (DDK) (Cdc7-Dbf4). Both CDK and DDK are activated in late G1 phase by the binding of unstable regulatory subunits, the Clb5,6 and Dbf4 proteins, respectively.

Both CDK and DDK are needed for Cdc45 protein to load DNA polymerases and other replication proteins onto origins during S-phase (Zou and Stillman 1998; Aparicio *et al.* 1999). DDK phosphorylates Mcm2 *in vitro* (and likely *in vivo*) as part of the MCM complex (Lei *et al.* 1997; Oshiro *et al.* 1999; Weinreich and Stillman 1999). Because the MCM complex is believed to act as the replicative helicase (Labib and Diffley 2001), it is thought that inhibition of the helicase activity is abolished by phosphorylation, perhaps by allosteric change of the MCM complex (Sclafani *et al.* 2002, 2004; Fletcher *et al.* 2003; Chen *et al.* 2005). Hence, both events are necessary for producing the binding of DNA replication proteins and fork movement (Jares *et al.* 2000; Lei and Tye 2001). The essential DDK step in DNA replication initiation can be bypassed in budding yeast cells carrying the *mcm5-bob1* mutation (Jackson *et al.* 1993; Hardy *et al.* 1997), which produces constitutive Cdc45 loading (Sclafani *et al.* 2002). In this regard, the *mcm5-bob1* encoded protein may mimic the allosteric change required for the MCM helicase to become an active complex (Sclafani *et al.* 2002, 2004; Fletcher *et al.* 2003; Chen *et al.* 2005). At the same time, additional CDK activity is still needed to initiate DNA replication. Thus, cells that carry either a *clb2*Δ or a *clb5*Δ mutation are ineffective in *mcm5-bob1*-dependent bypass of DDK (Sclafani *et al.* 2002). In this manner, *clb2*Δ and *clb5*Δ mutations suppress the *mcm5-bob1* mutation (Sclafani *et al.* 2002).

Concomitantly, DNA checkpoint mechanisms have evolved to monitor the successful completion of cell cycle events involving DNA replication and mitosis (see reviews in Foiani *et al.* 2000; Nyberg *et al.* 2002; Kastan and Bartek 2004). In *Saccharomyces cerevisiae*, Rad53 is an essential dual specificity protein kinase that is part of a signal transduction cascade involved in the response to DNA damage or stalled DNA replication forks. In this cascade, Mec1 and to a lesser extent Tel1 protein kinases

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activate Rad53 to prevent cell cycle progression, stabilize replication forks, and facilitate repair. Sensor proteins that bind Rad53 through its two fork head-associated (FHA) domains are needed either for the damage, *e.g.*, Rad9 (Sun *et al.* 1998a), or for the replication response, *e.g.*, Mrc1 (Alcasabas *et al.* 2001a). Mutations in both FHA1 and FHA2 domains of Rad53 ablate most checkpoint responses (Schwartz *et al.* 2003). Thus, Mec1 and Rad53 are needed for both responses, while Rad9 and Mrc1 are needed only for DNA damage or replication responses, respectively. These responses are important medically as defects in the human homologs of Rad53 (Chk2) or Mec1 (ATM/ATR) lead to human cancer by increasing genomic instability (Kastan and Bartek 2004).

The essential function of Rad53 or Mec1 kinases can be rescued by overexpression of the large subunit of ribonucleotide reductase encoded by *RNR1* or by downregulation of the Sml1 protein, which when bound to Rnr1 protein holds it in check (Sanchez *et al.* 1996; Desany *et al.* 1998; Zhao *et al.* 1998, 2000, 2001; Zhao and Rothstein 2002). Because an increase in deoxyribonucleotide levels can rescue loss of the *RAD53* or *MEC1* genes, an essential function of these kinases is to upregulate deoxyribonucleotide levels (Chabes *et al.* 2003). Failure to remove Sml1 in *rad53*Δ or *mec1*Δ mutants results in incomplete DNA replication, defective mitochondrial DNA propagation, decreased dNTP levels, and cell death (Zhao *et al.* 2001; Cha and Kleckner 2002).

Another one of Rad53's functions appears to be monitoring the stability of replication forks. *RAD53* mutants accumulate unusual replication structures at replication forks when treated with hydroxyurea (Lopes *et al.* 2001; Sogo *et al.* 2002). Replication fork catastrophe occurs in checkpoint mutants presented with DNA-damaging agents such as MMS (Tercero and Diffley 2001) or agents that stall replication such as HU (Sogo *et al.* 2002).

RAD53 is likely involved in S-phase progression control. Reduced origin firing was seen in a *rad53* mutant using 2D-gel analysis (Lopes *et al.* 2001). The firing of late origins is also advanced in *rad53* and *mec1* mutants (Santocanale and Diffley 1998), resulting in a late origin becoming early replicating (Shirahige *et al.* 1998). The accelerated S-phase is thought to be a consequence of inappropriate initiation events (Santocanale *et al.* 1999). Rad53 may also modulate origin firing on the basis of growth conditions to optimize the rate of S-phase progression (Sidorova and Breeden 2002). These results suggest that Rad53 controls the timing of DNA replication during normal cell growth.

Rad53 is also involved in the degradation of excess, nonnucleosomal soluble histones, which are shown to accumulate in *rad53*Δ *sml1*Δ strains (Gunjan and Verreault 2003). Because deletion of the major copy of the H3/H4 histone gene pair, *HHT2-HHF2*, suppresses some of the phenotypes of *rad53*Δ *sml1*Δ but not of *mec1*Δ *tel1*Δ *sml1*Δ mutants, excess histones may account for the slow growth and chromosome loss phenotype that occurs in the absence of Rad53. In a recent genomewide study of the DNA integrity network (Pan *et al.* 2006), it was suggested that Rad53 protein kinase might also play a more direct role in maintenance of chromatin structure. However, the role of the histone chaperones Asf1, Cac-1 (CAF1-3 genes), and Hir1/2 complexes (Kaufman *et al.* 1997; Tyler *et al.* 1999; Sharp *et al.* 2001) in this process is less clear (Gunjan and Verreault 2003; Quivy and Almouzni 2003; Pan *et al.* 2006). These *rad53* phenotypes are consistent with an effect on histone homeostasis and DNA replication independent of the DNA checkpoint or deoxynucleotide levels.

Our previous studies indicated a link between the initiation of DNA replication and Rad53 (Dohrmann *et al.* 1999). We found that *cdc7* and *rad53* mutations were synthetically lethal and that *rad53* mutants have reduced Dbf4 protein levels. Our original hypothesis was that this lethality results from reduced protein levels of Dbf4, the regulatory subunit of DDK. Consistent with this hypothesis, Rad53 binds (Dohrmann *et al.* 1999; Duncker *et al.* 2002) and phosphorylates Dbf4 (Weinreich and Stillman 1999; Kihara *et al.* 2000). However, we show in this report that the original hypothesis is incorrect and propose a new role for Rad53 in the initiation of DNA replication that is independent of Rad53's known roles in checkpoint or deoxynucleotide regulation.

MATERIALS AND METHODS

Yeast strains, media, and plasmids: Yeast strains and recombinant plasmids (Table 1) were grown and manipulated as described (Dohrmann *et al.* 1999; Sclafani *et al.* 2002; Pessoa-Brandao and Sclafani 2004). Standard genetic crosses were used for strain construction and tetrad analysis (Sherman *et al.* 1986; Burke *et al.* 2000).

To construct pPD339 and pPD342, pLGΔ178 (Guarente and Mason 1983) and pLG-ARS+ (Dowell *et al.* 1994), respectively, were subjected to partial digestion with *Hin*dIII to remove ~8000 bp containing the 2μ sequences. pPD357 was constructed by amplifying ARS1 sequences from pPD342 with primers ARS1-forward (5′ AAG CTT GCA TGC CTG CAG G 3′) and ARS1-reverse (5′ CCG CTC GAG AAT TCG AGC TCG GTA CCC 3′) digested with *Apa*I for integration at the *URA3* locus. PDY477 through PDY480 were also constructed by integrating the indicated ARS1-lacZ plasmids at the *URA3* locus.

The *RAD53*-activation domain fusion plasmid pPD233 (pACT2-lox-*RAD53*$_{AD}$) was constructed using the universal plasmid fusion system (Liu *et al.* 1998). Plasmid pRAS574 was constructed from plasmid pPD233 by replacement of a 1076-bp *Sac*I DNA restriction fragment from plasmid pPD335, which contains the *rad53-11* mutation, cloned by gap repair of *Sst*I-digested pPD94 (Dohrmann *et al.* 1999) using a *rad53-11* strain.

The *hht2-hhf2*Δ*::HIS3* (Mann and Grunstein 1992) construct was amplified by PCR using the "D" primers for each gene (Issel-Tarver *et al.* 2002) as the genes are divergently

transcribed. The resultant 2.4-kb PCR product was used to select His+ transformants. All *hht2-hhf2Δ::HIS3* strains were verified by use of the "A" and D primers of each gene, which yield no product, and the D primers, which yield a 2.4-kb product (Issel-Tarver *et al.* 2002).

Random spore analysis: *cyh2*^{R}*/CYH2*^{S} diploids were sporulated in liquid 0.3% potassium acetate (KAC) and the resultant asci were digested with snail glusulase at 10% for 30 min at room temperature (Dohrmann *et al.* 1999; Sclafani *et al.* 2002; Pessoa-Brandao and Sclafani 2004). The digested spores were washed extensively with distilled water and then diluted 1/50 to 1/100-fold and vortexed for 2 min. The dilutions were plated on selective medium that also contained cycloheximide to select for haploid progeny. The phenotype of the progeny was then tested by picking and patching the colonies to master plates and then replica plating. For diploid strain RSY1128 (Table 1), YPD plates with G418 (100 μg/ml) and cycloheximide (5 μg/ml) were used to select *rad53Δ::kanMX4 cyh2* colonies. As expected, all *rad53Δ::kanMX4* colonies also contained *sml1Δ::hygro* as *rad53Δ::kanMX4 SML1+* colonies are inviable (Zhao *et al.* 1998). In dropout medium, monosodium glutamate (1 g/liter) was substituted for $(NH_4)_2SO_4$ and G418 was used at 400 μg/ml.

Plasmid shuffle assays: For *LEU2* or *URA3* plasmid loss, at least 200 cells were plated on nonselective YPD plates and then the resultant colonies were replica plated to −Leu or −Ura dropout media to identify Leu$^-$ or Ura$^-$ clones, respectively. Alternatively, 5-fluoroorotic acid (5-FOA) was used to select Ura$^-$ colonies (Burke *et al.* 2000).

Minichromosome loss: Mitotic loss rates were determined (Lengronne and Schwob 2002) in cells carrying plasmids pDK247 (*LEU2 ADE3*) with one ARS or eight ARSs (pDK368-7) (Hogan and Koshland 1992) or plasmids bearing early- (p305.2), middle- (pARS1), or late-firing (p12) ARSs (Ferguson *et al.* 1991; Friedman *et al.* 1996). All loss rates are percentage per generation and were determined in triplicate.

One-hybrid and two-hybrid assays: Liquid culture β-galactosidase and X-Gal assays were done as previously described (Shellman *et al.* 1998; Dohrmann *et al.* 1999).

Rad53 protein immunoblots: Strains with a 3XHA-Rad53 construct (pPD328) integrated at the Rad53 locus were analyzed with anti-HA antibody as described (Pessoa-Brandao and Sclafani 2004).

RESULTS

Bypass of *cdc7* is dependent upon *RAD53*: The *mcm5-bob1* mutation bypasses the essential function of the DDK in that all *cdc7*ts and *dbf4*ts mutants are suppressed by *mcm5-bob1* (Tables 1 and 2) and *mcm5-bob1 cdc7Δ*, *mcm5-bob1 dbf4Δ*, and *mcm5-bob1 cdc7Δ dbf4Δ* mutants are viable (Jackson *et al.* 1993; Hardy *et al.* 1997; Sclafani *et al.* 2002). Bypass of DDK by *mcm5-bob1* also functions in all common genetic backgrounds that were tested (W303, A364a, and S288C—data not shown) (Weinreich and Stillman 1999). If the synthetic lethality of *cdc7 rad53* mutants is due to reduced Dbf4 protein levels, which would compromise the mutant *cdc7* protein kinase as proposed (Dohrmann *et al.* 1999), than *mcm5-bob1* should suppress it as it bypasses deletions of both essential *CDC7* and *DBF4* genes. We used a "plasmid sectoring assay" (Dohrmann *et al.* 1999) to test the idea. In this case, a *cdc7 rad53* strain is kept alive with a plasmid-borne copy of *CDC7*. The synthetic lethality can be monitored by a red/white colony-sectoring assay. In this case, strain PDY201 *cdc7 rad53* requires the *CDC7* plasmid for viability and demonstrates a red nonsectored colony morphology. If one complements the *cdc7 rad53* strain with either another *CDC7* or the *RAD53* plasmids, then the strain can lose *CDC7* plasmid pPD7, which results in a red/white sectored colony morphology. The *mcm5-bob1* mutation was introduced into strain PDY201 by transformation and recombination using plasmid pRAS490 (Table 1) (Fletcher *et al.* 2003; Pessoa-Brandao and Sclafani 2004), generating the triple-mutant strain PDY420 *cdc7 rad53 mcm5-bob1*. Surprisingly, the *cdc7 rad53 mcm5-bob1* strain was unable to lose the *CDC7* plasmid and demonstrated the nonsectoring colony morphology (100/100 clones tested). This indicates that *mcm5-bob1* is unable to suppress the *cdc7 rad53* synthetic lethality (Table 2).

One possible explanation for this result is that the *rad53* mutation suppresses *mcm5-bob1* bypass similarly to *clb5* or *clb2* mutations (Sclafani *et al.* 2002). When *mcm5-bob1* is suppressed, it can no longer suppress *cdc7*ts mutations, and thus the strains become temperature-sensitive mutant (Tsm)$^-$ again. To test the effect of a *rad53* mutation directly on *mcm5-bob1* bypass, we exploited the fact that the "leaky" *cdc7-4* allele is not synthetically lethal with *rad53* mutations (Weinert *et al.* 1994; Dohrmann *et al.* 1999) (Table 2). We crossed strain PDY258 *rad53-11 pep4Δ::URA3* with strain PDY253 *cdc7-4 mcm5-bob1*. Because the *pep4Δ::URA3* marker is tightly linked to the *rad53* locus (Dohrmann *et al.* 1999) and *pep4* mutations alone have no effect on *cdc7* or *mcm5-bob1* mutations (Jackson *et al.* 1993), it can be used to follow *rad53*. Because *CDC7* (chromosome IV) and *MCM5* (chromosome XII) are unlinked, the meiotic products of a single ascus will display predominantly 3+:1− Tsm phenotype segregation indicative of an extragenic suppressor (Hardy *et al.* 1997). Our results demonstrated a decrease of 3+:1− tetratype (T) asci (37 *vs.* 53.3 expected) and a concomitant increase of nonparental ditype (NPD) 2+:2− asci (36 *vs.* 13.3 expected; $P < 0.01$). As seen for *clb5* or *clb2* suppression (Sclafani *et al.* 2002), the increase in 2+:2− asci is the result of suppression of *mcm5-bob1*, which in this case causes a *cdc7 mcm5-bob1 rad53* strain to become Tsm$^-$. Indeed, a survey of 25 individual *cdc7 rad53* Tsm$^-$ colonies indicated that 11/25 (46%) contained the *mcm5-bob1* mutation as expected. The presence of *mcm5-bob1* was determined by backcrossing each spore clone to *cdc7*ts tester strains and checking whether the resulting *cdc7/cdc7 +/ mcm5-bob1* diploid papillated to Tsm$^+$ (Hardy *et al.* 1997). As *mcm5-bob1* is recessive, papillation results from the production of *mcm5-bob1/mcm5-bob1* homozygotes by mitotic recombination in 5–10% of the cells. Thus, while strain P138 carrying the *cdc7-4 mcm5-bob1* mutations is Tsm$^+$, strain PDY423 with the *cdc7-4 mcm5-bob1 rad53* mutations is Tsm$^-$ (Tables 1 and 2). Neither plasmid pPD348 (pGAP-RNR1) nor addition of the *sml1* mutation, both of which increase deoxyribonucleotide levels and bypass

TABLE 1

List of *S. cerevisiae* strains and recombinant plasmids used in this study

Strain or plasmid	Genotype	Source/reference/alias
PDY037	*MAT***a** *ura3 his3 leu2 cdc7Δ1::HIS3 mcm5-bob1-1 3HA-RAD53::URA3*	Pessoa-Brandao and Sclafani (2004)
PDY201	*MAT*α *cdc7-1 rad53-31 ade2 ade3 leu2 trp1 ura3* (pPD7 = YCp *CDC7-ADE3*)	Dohrmann *et al.* (1999)
PDY253	*MAT***a** *cdc7-4 ade2 ura3*	This study
PDY258	*MAT*α *rad53-11 pep4Δ::URA3 ura3*	
PDY294	*MAT*α *rad53Δ::hisG-URA3-hisG his3 lys2 leu2 trp1 ura3* (*pGAP-RNR1-2*μ *TRP*)	This study
PDY297	*MAT*α *rad53Δ::hisG his3 lys2 leu2 trp1 ura3* (*pGAP-RNR1-2*μ *TRP*)	This study
PDY376	*MAT***a** *ura3 leu2 trp1 3HA-RAD53::URA3*	Pessoa-Brandao and Sclafani (2004)
PDY420	*MAT*α *cdc7-1 mcm5-bob1-2 rad53-31 ade2 ade3 leu2 trp1 ura3* (pPD7 = YCp *CDC7-ADE3*)	Dohrmann *et al.* (1999)
PDY423	*MAT***a** *cdc7-4 rad53-11 pep4Δ::URA3 mcm5-bob1-1 ura3*	This study
PDY425	*MAT***a** *his3 cdc7-4 mcm5-bob1-2 ura3 rad53-11 pep4Δ::URA3*	Dohrmann *et al.* (1999)
PDY449	*MAT*α *rad53Δ::hisG URA3:: lacZ* vector *his3 lys2 leu2 trp1* (*pGAP-RNR1-2*μ *TRP*)	This study
PDY477	*MAT*α *rad53Δ::hisG URA3::ARS1$_{UAS}$-lacZ his3 lys2 leu2 trp1 ura3* (*pGAP-RNR1-2*μ *TRP*)	This study
PDY478	*MAT*α *rad53Δ::hisG URA3::1xF.ARS1$_{UAS}$ lacZ his3 lys2 leu2 trp1 ura3* (*pGAP RNR12*μ *TRP*)	This study
PDY480	*MAT***a** *leu2 trp1 rad53Δ::hisG URA3::ARS.2xR* (*pGAP-RNR1-2*μ *TRP*)	This study
P101	*MAT*α *leu2 his7 tyr1 cdc7-3 mcm5-bob1-1 lys2*	Jackson *et al.* (1993); Hardy *et al.* (1997)
P138	*MAT*α *leu2 ura3 cdc7-1 mcm5-bob1-1 cyh2*	Jackson *et al.* (1993); Hardy *et al.* (1997)
P253	*MAT***a** *ura3 can1 trp1 his3 cdc7-1 bob1-1 cyh2*	Jackson *et al.* (1993); Hardy *et al.* (1997)
P142	*MAT***a** *leu2 ura3 cdc7-1 mcm5-bob1-1*	Jackson *et al.* (1993); Hardy *et al.* (1997)
1815-4C	*MAT***a** *ura3 mad2-1*	P. Megee
RSY299	*MAT*α *his3 leu2 trp1 ura3*	Jackson *et al.* (1993); Hardy *et al.* (1997)
RSY 311	*MAT***a** *trp1 leu2 ura3 can1 his6 bar1*	Sclafani *et al.* (2002)
RSY465	*MAT***a** *leu2 ura3 trp1 his7 rad9Δ::LEU2*	7859-7-4 (T. Weinert)
RSY847	*MAT***a** *leu2 trp1 his3 ura3 cdc7-7 mcm5-bob1-2*	Pessoa-Brandao and Sclafani (2004)
RSY870	*MAT***a** *his3 trp1 mec1-1 sml1-1*	TWY304 (T. Weinert)
RSY1060	*MAT***a** *his3 leu2 trp1 ura3 sml1Δ::hygro rad53Δ::kanMX4*	JKT010 *rad53* (J. Tyler)[b]
RSY1064	*MAT***a** *leu2 trp1 rad53Δ::hisG URA3::ARS.2xR* (*pGAP-RNR1-2*μ *LEU2*)	This study
RSY1104	*MAT***a** *mrc1Δ::kanMX4 leu2 lys2 ura3 his3*	YCL060C (Open Biosystems)[c]
RSY1109	*MAT*α *cdc7-1 trp1-289 leu2 ura3 his3 mcm5-bob1-1 mrc1Δ::kanMX4*	This study[d]
RSY1111	*MAT*α *cdc7-1 trp1-289 leu2 ura3 his3 mrc1Δ::kanMX4*	This study[d]
RSY1113	*MAT***a** *chk1Δ::URA3 his3 leu2 trp1 ura3*	YS152 Sanchez *et al.* (1996)[b]
RSY1128	*MAT*α *leu2 ura3 cdc7-1 mcm5-bob1-1 cyh2* / *MAT***a** *leu2 ura3 + + + rad53Δ::kanMX4 his3 trp1 sml1Δ::hygro + + + +*	P138 X RSY1060 (This study)[a]
RSY1139	*MAT***a** *leu2 ura3 his3 cdc7-7 tel1Δ::kanMX4 trp1 mcm5-bob1-2*	This study[a]
RSY1141	*MAT*α *mec1-1 sml1-1 cdc7-1 leu2 his3 ura1 ura3 cyh2*	This study
RSY1142	*MAT***a** *cdc7-1 leu2 ura3 rad53Δ::kanMX4 his3 sml1Δ::hygro* (YCp *URA3 RAD53*)	This study[a]
RSY1143	*MAT***a** *cdc7-1 leu2 ura3 rad53Δ::kanMX4 his3 smlΔ1::hygro* (*pARS CEN URA3 LEU2 RAD53*)	This study[a]
RSY1145	*MAT*α *cdc7-1 mcm5-bob1-1 leu2 ura3 his3 rad53Δ::kanMX4 sml1Δ::hygro* (*pARS CEN URA3 RAD53*)	This study[a]
RSY1157	*MAT***a** *cdc7-1 leu2 ura3 rad53Δ::kanMX4 his3 sml1Δ::hygro hht2Δhhf2Δ::HIS3* (*pARS CEN URA3 RAD53*)	This study[a]
RSY1158	*MAT*α *cdc7-1 mcm5-bob1-1 leu2 ura3 his3 rad53Δ::kanMX4 sml1Δ::hygro hht2Δhhf2Δ::HIS3* (*pARS CEN URA3 RAD53*)	This study[a]

(*continued*)

TABLE 1

(Continued)

Strain or plasmid	Genotype	Source/reference/alias
RSY1161	*MAT*α *cdc7-1 mcm5-bob1-1 arg4 ura3 can1 cyh2 his3 ade1 clb5::ARG4 hht2Δhhf2Δ::HIS3*	This study
RSY1162	*MAT***a** *ade2 his3 leu2 trp1 ura3 asf1Δ::kanMX4*	JLY1017 (J. Tyler)[b]
RSY1163	*MAT***a** *ade2 his3 leu2 trp1 ura3 cac1Δ::LEU2*	JLY1018 (J. Tyler)[b]
RSY1164	*MAT***a** *tel1Δ::kanMX4 ura3 his3 leu2 met15*	YBL088C (Open Biosystems)[c]
RSY1177	*MAT***a** *cdc7-1 leu2 rad9Δ::LEU2 mrc1Δ::kanMX4*	This study[d]
RSY1179	*MAT*α *ura3 his3 lys2 leu2 dun1Δ::kanMX4*	YDL101C (Open Biosystems)[c]
RSY1195	*MAT*α *cdc7-1 mcm5-bob1-1 leu2 ura3 his3 rad53Δ::kanMX4 sml1Δ::hygro* (YCp *LEU2 RAD53*)	This study[a]
RSY1199	*MAT*α *leu2 his3 cdc7-1 cac1Δ::LEU2 ade2 ura3*	This study[a]
pPD1	pRS316-*CDC7* (*URA3*)	Dohrmann *et al.* (1999)
pPD7	pRS314-*CDC7-ADE3* (*TRP1*)	Dohrmann *et al.* (1999)
pPD60	pRS316-*RAD53*$^+$ (*URA3*)	Dohrmann *et al.* (1999)
pRS423	*HIS3* 2μ yeast shuttle vector	Christianson *et al.* (1992)
pGAP-RNR1	p*RNR1* from GAP pro (*TRP1*)	T. Weinert
pPD348	pRS423-GAP-*RNR1* (*HIS3*)	This study
pPD348	pRS425-GAP-*RNR1* (*LEU2*)	This study
pRAS490	*mcm5-bob1-2* (URA3)	Pessoa-Brandao and Sclafani (2004)
pDK243	CEN4 (1x ARS) (*LEU2*)	Hogan and Koshland (1992)
pDK368-7	CEN4 (8x ARS) (*LEU2*)	Hogan and Koshland (1992)
p305.2	ARS305 CEN5 (*URA3*)	Friedman *et al.* (1996)
pARS1	ARS1 CEN5 (*URA3*)	Friedman *et al.* (1996)
p12	ARS1412 CEN5 (*URA3*)	Friedman *et al.* (1996)
pLG-ARS1+	ARS1-lacZ one-hybrid vector (*URA3*)	Dowell *et al.* (1994)
pPD339	pLGΔ178 (*URA3*, Δ2μ seq)	Guarente and Mason (1983)
pPD342	pLG-ARS1+ (*URA3*, Δ2μ seq)	This study
pPD357	pLG-1xF.ARS1 (*URA3*, Δ2μ seq)	This study
pPD358	pLG-1xR.ARS1 (*URA3*, Δ2μ seq)	This study
pPD359	pLG-2xR.ARS1 (*URA3*, Δ2μ seq)	This study
pGAD-2F	p*GAL4*$_{AD}$ (*LEU2*)	Chien *et al.* (1991)
pUNI10	Univector Plasmid System	Liu *et al.* (1998)
pACT2-lox	*GAL4*$_{AD}$-fusion vector	Liu *et al.* (1998)
pPD206	pUNI10-*RAD53*$^+$	This study
pPD233	pACT2-lox-*RAD53*$_{AD}$(*LEU2*)	This study
pRS315	*LEU2* yeast shuttle vector	Sikorski and Hieter (1989)
pPD361	pRS315-*RAD53* (*LEU2*)	This study
pY2	p*GAL4*$_{DB}$ (*TRP1*)	Sadowski *et al.* (1992)
pPD94	p*GAL4*$_{DB}$-*RAD53* (*TRP1*)	Dohrmann *et al.* (1999)
pRS316-RAD53	*RAD53*$^+$ (*URA3*)	Sun *et al.* (1996, 1998a,b)
pRS316-rad53kd	*rad53* K227A D339A (*URA3*)	Sun *et al.* (1996)
pRS316-rad53-NVS	*rad53* NVS/AAA (655-657) (*URA3*)	Sun *et al.* (1998a,b)
pRS316-rad53-H622A	*rad53* H622A (*URA3*)	Sun *et al.* (1998a,b)
pRS315-RAD53	*RAD53*$^+$ (*LEU2*)	Schwartz *et al.* (2003)
pRS315-rad53-fha1	*rad53* R70A N107A (*LEU2*)	Schwartz *et al.* (2003)
pRS315-rad53-fha1,2	*rad53* R70A N107A NVS-AAA (655–657) (*LEU2*)	Schwartz *et al.* (2003)
pJG46	*B42*$_{AD}$ (*TRP1*) vector	Duncker *et al.* (2002)
pRAD53	B42$_{AD}$-RAD53$^+$ (*TRP1*)	Duncker *et al.* (2002)
FHA1	B42$_{AD}$-rad53(I4-S165) (*TRP1*)	Duncker *et al.* (2002)
KIN	B42$_{AD}$-rad53(G177-N599) (*TRP1*)	Duncker *et al.* (2002)
FHA2	B42$_{AD}$-rad53(M497-S821) (*TRP1*)	Duncker *et al.* (2002)
fha1	B42$_{AD}$-rad53(I4-S165; R70A) (*TRP1*)	Duncker *et al.* (2002)
fha2	B42$_{AD}$-rad53(M497-S821; R605A) (*TRP1*)	Duncker *et al.* (2002)
pRM102	Gal10p-HHT2 Gal1p-HHF2 (*URA3*)	Mann and Grunstein (1992)

All yeast strains are congenic with A364a except if indicated otherwise:
[a] W303/A364a hybrid.
[b] W303.
[c] S288C.
[d] S288C/A364a hybrid.

TABLE 2

Summary of phenotypes of *cdc7*, *rad53*, *mcm5-bob1*, and *hht2-hhf2* strains

Genotype	Viability	Tsm[a]
cdc7-1, *cdc7-4*	+	−
rad53-11 or *rad53*Δ *sml1*Δ	+	+
cdc7-1 rad53-11 or *cdc7-1 rad53*Δ *sml1*Δ	−	NA[c]
cdc7-x[b] *mcm5-bob1*	+	+
cdc7-1 rad53-11 mcm5-bob1 or *cdc7-1 rad53*Δ *sml1*Δ	−	NA[c]
*cdc7-1 rad53*Δ *sml1*Δ *hht2-hhf2*Δ	+	−
*cdc7-1 rad53*Δ *sml1*Δ *mcm5-bob1 hht2-hhf2*Δ	+	+
cdc7-4 rad53-11	+	−
cdc7-4 rad53-11 mcm5-bob1	+	−
*cdc7-1 mcm5-bob1 clb5*Δ	+	−
*cdc7-1 mcm5-bob1 clb5*Δ *hht2-hhf2*Δ	+	−

[a] Temperature sensitivity.
[b] *x*, any allele including *cdc7*Δ.
[c] Not applicable.

a *rad53*Δ (Sanchez *et al.* 1996; Desany *et al.* 1998; Zhao *et al.* 1998), had any effect (Table 2 and data not shown). We conclude that bypass or suppression of *cdc7* by *mcm5-bob1* is dependent upon *RAD53* and this effect is independent of deoxyribonucleotide levels.

The triple-mutant strain PDY425 *cdc7-4 mcm5-bob1 rad53* was temperature sensitive (Table 2) and arrested as large-budded cells (>85%) after 4 hr at the restrictive temperature. The majority of these large-budded cells (>90%) had the nucleus at the mother-bud neck (data not shown). This terminal phenotype is similar to that of *cdc7* mutants (Hartwell 1973). Because *mcm5-bob1* is suppressed by a *rad53* mutation and cannot suppress the *cdc7*ts mutation, a *cdc7*ts and a *cdc7*ts *mcm5-bob1 rad53* strain have the same phenotype (Table 2).

Rad53 kinase activity but neither FHA domain is needed for both *cdc7* viability and *mcm5-bob1* suppression: Our previous results (Dohrmann *et al.* 1999) and those above used *rad53* hypomorphic alleles. We produced a series of strains that had either *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro* or *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro mcm5-bob1* genotypes (Table 1) and a complementing *RAD53*$^+$ plasmid by performing random spore analysis with diploid strain RSY1128 (Table 1). Resultant *cdc7*ts segregants could be identified as Tsm$^-$ and *cdc7*ts *mcm5-bob1* segregants as Trp$^+$ Tsm$^+$ because *cdc7*ts is tightly linked to *TRP*1$^+$ (2 cM). Because *CDC7* and *MCM5* are unlinked, 50% of *TRP1*$^+$ colonies will be Tsm$^+$ because *mcm5-bob1* suppresses *cdc7*ts (Hardy *et al.* 1997). If RSY1128 alone or RSY1128 with a URA3 vector pRS316 was used no *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro* or *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro mcm5-bob1* isolates were obtained in 100 *rad53*Δ*::kanMX4 sml1*Δ*::hygro* colonies analyzed. If a *URA3 RAD53* complementing plasmid was included and also selected in the spores, the expected 50:50 ratio of *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro* and *cdc7 rad53*Δ*::kanMX4 sml1*Δ*::hygro mcm5-bob1* genotypes was obtained. The presence of *mcm5-bob1* in these strains was also confirmed by complementation test with *cdc7* tester strains (Hardy *et al.* 1997; Sclafani *et al.* 2002). These results indicate that *cdc7-1* is synthetically lethal with *rad53*Δ and this lethality cannot be bypassed by *mcm5-bob1*.

To test additional *rad53* mutations, we used a "plasmid shuffle" assay with *rad53*Δ *sml1*Δ *cdc7* (pRAD53) strain RSY1142 and *rad53*Δ *sml1*Δ *cdc7 mcm5-bob1* strain RSY1145 obtained from this cross or by "shuffle" of these strains with a *LEU2 RAD53* plasmid to produce strains RSY1143 and RSY1195 (Table 1). Different *rad53* mutant plasmids (Tables 1 and 3) were transformed into these strains and tested for complementation by determining if the original pRAD53 plasmid could be lost.

TABLE 3

Plasmid "shuffle" assay with *rad53* mutations in *cdc7*ts and *cdc7*ts *mcm5-bob1* strains

Plasmid for "shuffle"	Mutant domain(s)	*cdc7*ts[a]	*cdc7*ts *mcm5-bob1*[a,b] 22°	36°
Vector	NA	−	−	−
RAD53$^+$	None	+	+	+
rad53 R70A N107A	FHA1	+	+	+
rad53 NVS/AAA	FHA2	+	+	+
rad53 H622A	FHA2	+	+	+
rad53 K227A D339A	Kinase	−	−	−
rad53 R70A N107A NVS/AAA	FHA1 FHA2	+	+	+

[a] Strains RSY1142 and RSY1143 *rad53*Δ *sml1*Δ *cdc7* (pRAD53) were transformed with the indicated plasmids (Table 1). + or − indicates whether the initial pRAD53 plasmid could or could not be shuffled and replaced with the listed plasmid, respectively, at the permissive temperature of 22°.
[b] RSY1145 and RSY1195 *rad53*Δ *sml1*Δ *cdc7 mcm5-bob1* (pRAD53) were transformed with the indicated plasmids (Table 1). + or − indicates whether the resultant *rad53*Δ *sml1*Δ *cdc7 mcm5-bob1* strain with the shuffled plasmid listed could grow at the restrictive temperature of 36°. Growth at 36° indicates *mcm5-bob1* suppression of *cdc7*ts.

TABLE 4

Only *RAD53* is required for both *cdc7* viability and *mcm5-bob1* suppression

Cross	Query gene	Tsm$^+$ (*cdc7*ts *mcm5-bob1*)	Tsm$^-$ (*cdc7*ts)	*n*
RSY311 × P101	None	50	50	34
RSY1060 × P138	*rad53*	0	0	48
P142 × RSY870	*mec1*	59	41	22
RSY1164 × 847	*tel1*	58	42	26
RSY1113 × P138	*chk1*	50	50	43
P253 × RSY1104	*mrc1*	42	58	27
RSY847 × 1815-4C	*mad2*	41	59	20
RSY1139 × RSY1141	*tel1 mec1*	42	58	20
RSY1111 × RSY465	*rad9*	45	55	20
RSY1179 × P253	*dun1*	48	52	21
JLY1018 × P142	*asf1*	53	47	17
JLY018 × P142	*cac1*	50	50	24
RSY1111 × RSY465	*rad9 mrc1*	—	40	20
RSY1109 × RSY1177	*rad9 mrc1*	43	—	23

All numbers are the percentage of colonies with the query mutant gene(s) that also have *cdc7 mcm5-bob1* or just *cdc7* mutations. Because all genes are unlinked, 50% of colonies with the *cdc7* mutation will be Tsm$^-$. Tsm$^+$ *cdc7* colonies contain *mcm5-bob1* (Hardy *et al.* 1997); *n*, number of colonies in tetrads examined. *cdc7*ts mutation was followed by the Tsm$^-$ phenotype or by linkage to *TRP1*.

Mutations in the FHA1 domain (R70A N107A), in the FHA2 domain (NVS/AAA and H622A), or in both the FHA1 and the FHA2 domains (R70A N107A NVS/AAA) (Sun *et al.* 1998b; Schwartz *et al.* 2003) complemented (Table 3). Only the *rad53* K227A D339A kinase dead mutant failed to complement. All these *rad53* mutants except for the kinase-dead allele also complemented the *rad53*Δ *sml1*Δ *cdc7 mcm5-bob1* strains. In addition, they became Tsm$^+$, indicating that *mcm5-bob1* function was also restored. We conclude that Rad53 protein kinase activity but not its ability to mediate DNA checkpoint signaling via the two FHA domains (Pike *et al.* 2003; Schwartz *et al.* 2003) is needed for both *cdc7* viability and *mcm5-bob1* suppression.

Mutations in most other checkpoint genes have no effect on *cdc7* mutant viability and *mcm5-bob1* suppression: We determined whether the DNA replication, damage, or mitotic checkpoints are important for the effects we have observed by crossing different checkpoint mutants with a *cdc7*ts *mcm5-bob1* strain (Tables 1 and 4). Again, we used the tightly linked *TRP1* marker to identify *cdc7 mcm5-bob1* strains. Our results using tetrad analysis show that in contrast to the results with *rad53* mutants, both *cdc7* (Tsm$^-$) and *cdc7 mcm5-bob1* (Tsm$^+$) strains containing null mutations in *mec1*, *tel1*, *chk1*, *mrc1*, *rad9*, *dun1*, and *mad2* were easily obtained (Tables 1 and 4). Furthermore, all *cdc7 mcm5-bob1* strains with these checkpoint mutations were Tsm$^+$, indicating that *mcm5-bob1* bypass of *cdc7* was not affected. Similar results were also found for *mec1 tel1* and *rad9 mrc1* strains, which are defective in all DNA replication and damage checkpoint signaling (Sanchez *et al.* 1996; Alcasabas *et al.* 2001b). We conclude that loss of the DNA checkpoint *per se* in *rad53* mutants is not responsible for the effects we have observed.

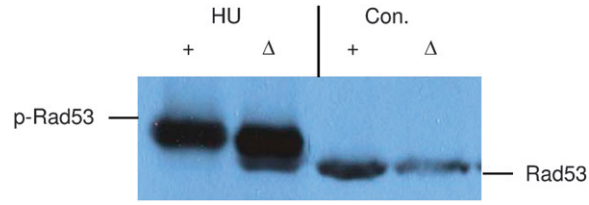

Figure 1.—Rad53 protein kinase is activated by HU even in the absence of Cdc7 protein. Wild-type (+) or *cdc7*Δ *mcm5-bob1* (Δ) cells with the tagged 3XHA-RAD53 gene were treated with 200 mm HU for 3.5 hr. Untreated control log-phase cells (Con.) were also examined. Immunoblots were prepared from cell extracts and probed with anti-HA antibody to detect the 3XHA-Rad53-tagged protein. The positions of the Rad53 (Rad53) and activated phosphorylated Rad53 (p-Rad53) proteins are indicated.

Rad53 can be activated by HU in the absence of Cdc7: Our results indicate that DDK's role is compromised in the absence of Rad53. Conversely, we tested if Rad53 function was compromised by the absence of DDK. Previously, we found that in *cdc7*Δ *mcm5-bob1* strains, the DNA damage checkpoint is intact and Rad53 can be activated by UV-induced DNA damage (Pessoa-Brandao and Sclafani 2004). The DNA replication checkpoint is also intact in these strains in that survival is high even when DNA replication is blocked with HU (Weinreich and Stillman 1999). We tested whether Rad53 can be activated in the absence of Cdc7 protein when DNA replication is blocked with HU (Figure 1). When Rad53 is activated, slower-migrating forms appear in the gel due to autophosphorylation and phosphorylation by Mec1 kinase (Pellicioli *et al.* 2001). Cells were arrested in S-phase with HU at 200 mm for 3.5 hr. As we have seen with UV damage, Rad53 protein can be activated by HU treatment in a *cdc7*Δ *mcm5-bob1* strain. However, the response is partially attenuated with somewhat less Rad53 protein being shifted. The amount of Rad53 protein that is activated must be sufficient because these *cdc7*Δ *mcm5-bob1* cells remain viable in HU even after 12 hr as seen for wild-type cells (data not shown). Therefore, Rad53 kinase can function in the DNA checkpoint even in the absence of DDK.

***rad53*Δ mutants demonstrate a minichromosome loss phenotype:** Because DDK is important for the initiation of DNA replication, we tested Rad53's role in this process. Many mutations in the genes involved in the initiation of DNA replication such as *mcm*s and *orc*s show a minichromosome loss phenotype (Maine *et al.* 1984) that is suppressible by addition of multiple ARS elements (Hogan and Koshland 1992; Foss *et al.* 1993; Loo *et al.* 1995; Hardy 1996). Strain PDY256 *RAD53*$^+$ or

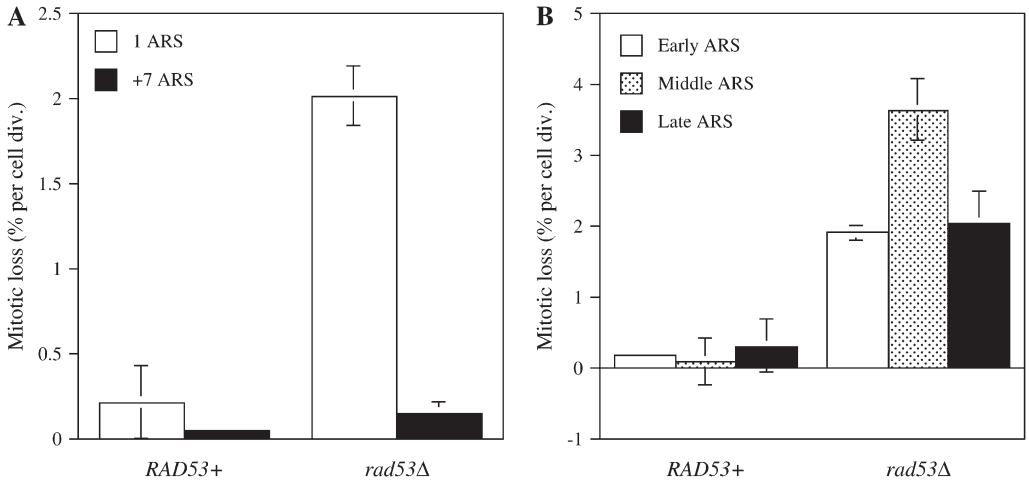

FIGURE 2.—*rad53*Δ mutants exhibit a minichromosome loss phenotype. (A) Mitotic loss rates were measured, as in MATERIALS AND METHODS, in wild-type (PDY256) and *rad53*Δ (PDY294) cells that were transformed with either pDK243 (1 ARS) or pDK368-7 (+7 ARS). Shown are mean values from three independent experiments. (B) Mitotic rates were measured as in A for WT (PDY256) and *rad53*Δ (PDY294) cells transformed with one of three plasmids: p305.2 (early-firing ARS305), pARS1 (early/middle-firing ARS1), or p12 (late-firing ARS1412).

PDY294 *rad53*Δ was transformed with plasmids containing either one ARS (pDK243) or eight ARSs (pDK368-7). Mitotic loss rates were calculated by fluctuation tests and were found elevated 20-fold in the *rad53*Δ strain (Figure 2A). Also as seen in other initiation mutants, the loss rate could be suppressed completely by the addition of multiple ARS elements.

The minichromosome loss phenotype was not limited to just ARS1. We tested the minichromosome loss phenotype on individual plasmids that carry individual ARSs that fire at different times during S-phase: ARS305 (early firing), ARS1 (early/middle firing), and ARS1412 (late firing) (FERGUSON *et al.* 1991; FRIEDMAN *et al.* 1996). In each case, elevated minichromosome loss phenotypes were seen in the *rad53*Δ mutant strain (Figure 2B).

Rad53 is targeted to ARS1 through its kinase domain: Due to the increased minichromosome loss rate in *rad53*Δ strains, we hypothesize that Rad53 protein may bind to origins of replication. Binding of Rad53 protein to origins by chromatin immunoprecipiation (ChIP) has been shown (KATOU *et al.* 2003). However, this binding was barely detectable as compared to Mrc1 or Tof1 proteins and required checkpoint activation. Therefore, we used the one-hybrid system as an alternative. This strategy has been used successfully to detect proteins that interact with a yeast origin *in vivo*, including Orc6 and Dbf4 proteins (LI and HERSKOWITZ 1993; DOWELL *et al.* 1994). Briefly, the reporter gene lacZ is fused downstream of a protein binding site in the promoter region. Hybrid proteins containing transcriptional activation domains (AD) that recognize this site, either directly or indirectly, can activate transcription of the reporter gene. In this case, the yeast ARS1 origin of replication was fused in front of the lacZ reporter (DOWELL *et al.* 1994) and integrated in single copy at the *URA3* locus. Because overexpression of *RAD53*-AD from the constitutive *ADH* promoter dramatically inhibits cell growth in *RAD53*+ strains (SUN *et al.* 1996), these experiments were carried out in *rad53*Δ reporter strains PDY477, PDY478, and PDY480 (Table 1). Several reporters were tested with one or two copies of ARS1 in different orientations (for example, *1xF.ARS1*$_{UAS}$ indicates one copy of ARS1 in the forward orientation) (Figure 3A). Only the *RAD53*-AD fusion activated transcription two- to fourfold above background in three different ARS1-lacZ reporter strains (1xF, 1xR, and 2xR). A *RAD53* construct lacking the activation domain (pPD361; Table 1) failed to activate transcription, similar to the vector alone control. Only background activity was also detected with the *RAD53*-AD fusion in a control strain PDY449 (Table 1) with only the *lacZ* vector (data not shown). We conclude that Rad53, like Orc6 and Dbf4, can be targeted to the ARS1 origin of replication.

We determined the domain of Rad53 that binds ARS1-lacZ by using plasmids with different fragments of Rad53 fused to the B42 transcriptional AD (DUNCKER *et al.* 2002), using reporter strain RSY1064 (Table 1). All these plasmids produce Rad53-AD fusion proteins in similar amounts (DUNCKER *et al.* 2002). We tested full-length *RAD53*$^{+}$, kinase domain (G177-N599), and FHA1 (I4-S165) and FHA2 (M497-S821) domain fusions as well as the FHA1 (R70A) and FHA2 mutants (R605A) (Figure 4). We used the more sensitive X-Gal plate assay. The strongest signal was obtained with the Rad53 kinase domain fusion, while reduced signal was seen with the FHA1 domain and the full-length RAD53^{+} fusion. The FHA1 signal was ablated by the R70A mutation, which destroys binding to Dbf4 protein (DUNCKER *et al.* 2002).

We also tested the *rad53-11* mutation, which is also known as *mec2-1*, as it is checkpoint defective (WEINERT *et al.* 1994), has undetectable kinase activity *in vivo* (SUN *et al.* 1996) and *in vitro* (SIDOROVA and BREEDEN 2003), is synthetically lethal with *cdc7*, and suppresses

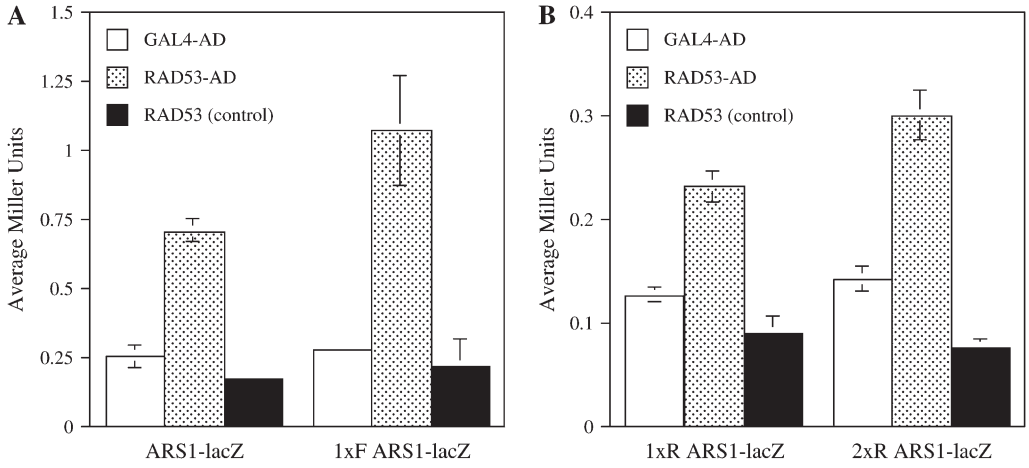

FIGURE 3.—Rad53 is targeted to origin of replication ARS1. (A and B) Quantitative liquid β-galactosidase assays. The indicated activation domain plasmids *GAL4*-AD (pGAD-2F), *RAD53*-AD (pPD233), and *RAD53* (pPD361 control without AD) were transformed into reporter strains PDY477 and PDY478 containing indicated versions of the integrated ARS1-lacZ reporter (Table 1). *1xF.ARS1*$_{UAS}$ indicates one copy of ARS1 in the forward orientation (MATERIALS AND METHODS), while *1xR.ARS1*$_{UAS}$ and *2xR.ARS1*$_{UAS}$ indicate one or two copies of ARS1 in forward or reverse orientation, respectively. Assays shown are mean values from three independent colonies.

mcm5-bob1 (Tables 1 and 2). We cloned and sequenced this allele (MATERIALS AND METHODS). A point mutation at position 1958 in the coding region results in a single-amino-acid change, G653E. This Rad53-11 fusion protein is still able to bind ARS1 (Figure 4). One possibility is that the Rad53-11 mutant protein can still bind the origin but is unable to phosphorylate an important substrate or that kinase activity is not important for origin binding. We conclude that Rad53 binds the ARS1 origin mainly through its kinase domain. Because our reporter strains are *rad53*Δ (Table 1), ARS1 binding by the Rad53 kinase domain fusion is checkpoint independent.

The Histone H3/H4 deletion suppresses *rad53* effects on *cdc7* and *mcm5-bob1*: Partial suppression of the *rad53*Δ phenotype by deletion of the major copy of the histone H3/H4 gene pair, *HHT2-HHF2*, has been shown (GUNJAN and VERREAULT 2003). The suppression results in increased resistance to low levels of HU (3 mM), an increased growth rate, and a reduction in chromosome loss rate of *rad53*Δ *sml1*Δ strains. These effects are similar to the effects of *rad53* mutations on *cdc7* viability and *mcm5-bob1* suppression described herein in that they are both dependent on Rad53 kinase activity but are independent of *mec1*, *mrc1*, *mec1 tel1*, and *sml1* null mutations and hence checkpoint function. Furthermore, neither deletion of *HHT2-HHF2* (GUNJAN and VERREAULT 2003) nor the *mcm5-bob1* mutation (DOHRMANN *et al.* 1999) can suppress the lethality of a *rad53*Δ. Therefore, a *hht2-hhf2*Δ*::HIS3* deletion (MANN and GRUNSTEIN 1992) was produced by transformation of both *cdc7 sml1*Δ *rad53*Δ (pRAD53 URA3) strain RSY1142 and *cdc7 mcm5-bob1 sml1*Δ *rad53*Δ (pRAD53 URA3) strain RSY1145 to His$^+$. These strains cannot lose the complementing *RAD53* plasmid because of the *cdc7-rad53* synthetic lethality (Tables 2 and 3). If suppression of *rad53*Δ occurs then both strains will be able to lose the pRAD53 URA3 plasmid and become resistant to 5-FOA, that is, become Ura−. This is indeed the case (Figure 5; Tables 1 and 2). In addition, the resultant *cdc7 mcm5-bob1 rad53*Δ *hht2-hhf2*Δ*::HIS3* strain RSY1158 also becomes Tsm$^+$, indicating that *mcm5-bob1* suppression of *cdc7* also is rescued by the H3/H4 deletion (Table 2). Thus, deletion of the major histone H3/H4 gene pair suppresses the effect of *rad53*Δ on *cdc7* mutant viability and *mcm5-bob1* suppression. The effect is specific to *rad53* suppression as *hht2-hhf2*Δ*::HIS3* does not suppress the effect of *clb5*Δ on *mcm5-bob1* in strain RSY1161 (Table 2) (SCLAFANI *et al.* 2002).

To further analyze the effect of the H3/H4 deletion, *cdc7*ts *rad53*Δ *hht2-hhf2*Δ*::HIS3* strain RSY1157 and *cdc7*ts *mcm5-bob1 rad53*Δ *hht2-hhf2*Δ*::HIS3* strain RSY1158 were grown at both restrictive (36°) and permissive (22°) temperatures (Figure 6). The DNA content was analyzed by flow cytometry (OSTROFF and SCLAFANI 1995). As expected for loss of DDK function

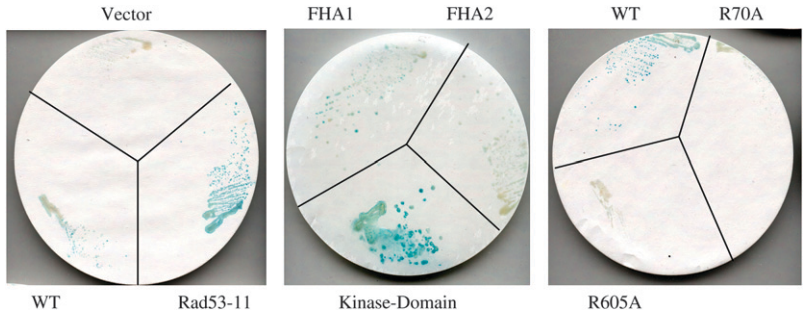

FIGURE 4.—Rad53 kinase domain binds ARS1. Strain PDY480 with an ARS-lacZ reporter containing different Rad53-AD fusions was assayed for β-galactosidase activity using the X-Gal filter assay. Plates were grown for 3 days and filters were developed for similar times. Fusions used were full-length wild-type Rad53 protein (821 residues), full-length Rad53-11 mutant (G653E), kinase domain (G177-N599), FHA1 (I4-S165), FHA2 (M497-S821), and mutant FHA1-R70A and mutant FHA2-R605A domains.

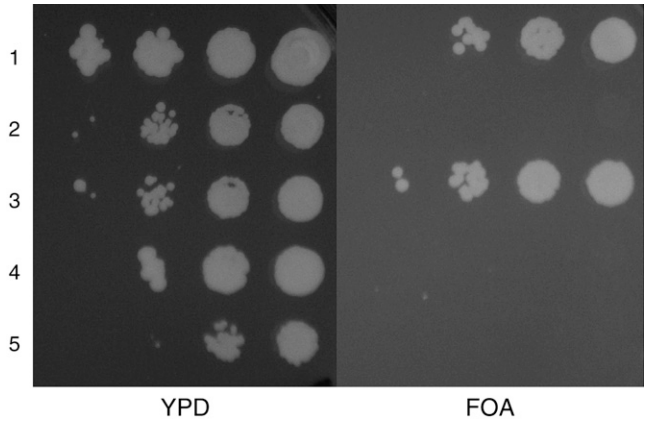

Figure 5.—Deletion of histone H3/H4 gene pair 2 suppresses the *cdc7-rad53* synthetic lethality. Strain RSY1142 *cdc7 rad53 sml1 his3 ura3* (pRAD53 URA3) (rows 2 and 5) or RSY1145 *cdc7 rad53 sml1 mcm5-bob1 his3 ura3* (pRAD53 URA3) (row 4) cells were transformed with a *hht2-hhf2Δ::HIS3* PCR fragment to His+ to produce strains RSY1157 (row 3) and RSY1158 (row 1), respectively. Colonies were diluted serially in 10-fold increments and spotted on either YPD or 5-FOA plates. Only cells with the *hht2-hhf2Δ::HIS3* deletion (rows 1 and 3) can lose the pRAD53 URA3 plasmid and grow on the 5-FOA plates after incubation at 22° for 3 days.

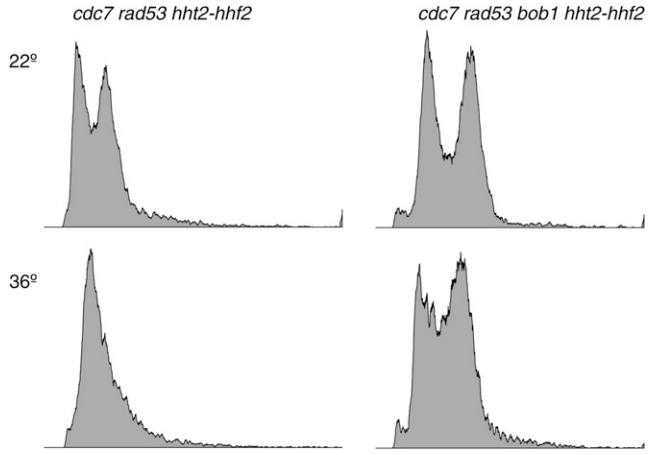

Figure 6.—Flow cytometry of the DNA content of *cdc7 rad53* mutant cells suppressed by histone H3/H4 deletion. Strains RSY1157 *cdc7*ts *rad53Δ hht2-hhf2Δ::HIS3* and RSY1158 *cdc7*ts *mcm5-bob1 rad53Δ hht2-hhf2Δ::HIS3* were grown at both restrictive (36°) and permissive (22°) temperatures in YPD medium for 4 hr and then processed for flow cytometry (Ostroff and Sclafani 1995). *y*-axis is cell number and *x*-axis is DNA content.

(Ostroff and Sclafani 1995; Sclafani 2000), the *cdc7*ts *rad53Δ hht2-hhf2Δ::HIS3* strain arrested at the G1/S boundary with a G1 content of DNA while the *cdc7*ts *mcm5-bob1 rad53Δ hht2-hhf2Δ::HIS3* strain continued to cycle, yielding both S and G2/M peaks. However, these *cdc7*ts *mcm5-bob1* cells progress into S-phase slower at the restrictive temperature because *mcm5-bob1* bypass of *cdc7* is inefficient (Weinreich and Stillman 1999; Pessoa-Brandao and Sclafani 2004). Because the cells have the expected phenotypes, the *hht2-hhf2Δ::HIS3* histone deletion suppresses all the observed effects of *rad53Δ* on *cdc7* viability and *mcm5-bob1* bypass.

DISCUSSION

In this report, we provide both genetic and molecular evidence that Rad53 kinase and DDK interact to regulate the initiation of DNA replication independently of Rad53's role in the DNA damage or replication checkpoints (Tables 2–4). In contrast, DDK has little if any role in the DNA checkpoint as Rad53 kinase can be activated by HU in the absence of DDK (Figure 1) and both checkpoints remain intact in *cdc7Δ mcm5-bob1* strains (Weinreich and Stillman 1999; Pessoa-Brandao and Sclafani 2004). We have demonstrated that Rad53 kinase activity is required for the viability of *cdc7* mutants and for *mcm5-bob1* to bypass the function of DDK (Table 3). Moreover, cells lacking Rad53 exhibit minichromosome loss defects that are suppressible by increased origin dosage (Figure 2), similar to known initiation mutants (Hogan and Koshland 1992; Foss *et al.* 1993; Loo *et al.* 1995; Hardy 1996). Finally, through one-hybrid experiments, we have demonstrated that Rad53 can be targeted to the ARS1 origin of DNA replication, mainly through its kinase domain (Figure 3 and 4).

We propose that Rad53 can bind to an origin independently of Dbf4 protein. The Rad53 kinase domain, which does not bind Dbf4 protein (Duncker *et al.* 2002), is the most effective at binding ARS1 (Figure 4). Rad53 kinase activity is needed for both *cdc7* viability and *mcm5-bob1* bypass (Table 4). In contrast, mutations in both FHA1and FHA2 domains of Rad53 have no effect (Table 3). These double FHA1 and FHA2 mutants have been shown to abolish Rad53 activation either by DNA replication inhibitors or by DNA-damaging agents and to ablate downstream checkpoint functions (Pike *et al.* 2003; Schwartz *et al.* 2003). Yet they have no effect on *cdc7* mutant viability or *mcm5-bob1* suppression (Table 3). Similarly, mutations in genes required for all aspects of the DNA checkpoint also have no effect (Table 4). These results demonstrate that neither the DNA damage nor the replication checkpoint response is required for Rad53's function in this context.

We can also rule out the hypothesis that in *cdc7* and *cdc7Δ mcm5-bob1* mutants replication is defective in some manner leading to stalled replication forks or DNA damage and that the DNA checkpoint "saves" these cells either by stabilizing the stalled forks or by preventing the cells from entering mitosis before S-phase is complete. Even *mec1 tel1* double mutations, which eliminate both DNA damage and replication checkpoints (Nyberg *et al.* 2002) have no effect. Previous studies also showed that *cdc7-1 mec1Δ* double mutants were viable (Desany *et al.* 1998).

Furthermore, there are not enough damage or stalled replication forks in the *cdc7*Δ *mcm5-bob1* mutant to constitutively activate Rad53 (Figure 1) (Pessoa-Brandao and Sclafani 2004). In fact, the *cdc7*Δ *dbf4*Δ *mcm5-bob1*mutant is sensitive to DNA damage because of a lack of translesion synthesis from the Rev3 (DNA pol ζ) pathway and not because of a defective DNA damage checkpoint (Pessoa-Brandao and Sclafani 2004). Therefore, DDK is not an important target of checkpoint control in *S. cerevisiae*, although it could be in other eukaryotes including fission yeast (Takeda *et al.* 1999), Xenopus (Costanzo *et al.* 2003), and humans (Dierov *et al.* 2004). However, recent results using Xenopus extracts have shown that DDK is not important in DNA damage checkpoint control (Petersen *et al.* 2006).

We propose that Rad53 through its kinase activity helps to stabilize replication complexes at origins during initiation. In the absence of Rad53, more DDK activity is needed for initiation, which can explain the *cdc7-rad53* synthetic lethality. Furthermore, Mcm5-bob1 protein, which may act by pushing out the A domain of Mcm5 to attract Cdc45 protein (Sclafani *et al.* 2002, 2004; Fletcher *et al.* 2003; Chen *et al.* 2005), is inefficient and may fail when the initiation complex is compromised by a lack of Rad53 function. This hypothesis is similar to one we proposed to explain the suppression of *mcm5-bob1* by loss of Cdk1-Clb5 kinase (Sclafani *et al.* 2002), a known regulator of initiation (Zou and Stillman 1998). In this scenario, reduced initiation at ARS1 occurs on the minichromosome (Figure 2) because initiation complexes are unstable or infrequently activated. By increasing the number of origins, "mass action" results in the activation of at least one active origin, thereby suppressing the defect.

Because *rad53*Δ *sml1*Δ strains grow slower than *mec1*Δ *sml1*Δ strains (Zhao *et al.* 2001; Gunjan and Verreault 2003) and histone suppression occurs only for *rad53*Δ *sml1*Δ strains (Gunjan and Verreault 2003), there must be a *mec1*-independent function of Rad53 that is important for cell cycle progression and does not involve the checkpoint function (Zhao *et al.* 2001; Gunjan and Verreault 2003). A Rad53-dependent surveillance mechanism that balances the rate of DNA replication and histone synthesis during S-phase and in response to DNA damage has been proposed to explain these effects (Gunjan and Verreault 2003). Rad53 may be involved in the degradation of excess soluble histones as these accumulate in *rad53*Δ *sml1*Δ strains and a reduction of H3/H4 levels with *hht2-hhf2*Δ mutation partially suppresses some *rad53* mutant phenotypes. We find that the *hht2-hhf2*Δ mutation also suppresses the effect of *rad53*Δ on both *cdc7* mutant viability and *mcm5-bob1* suppression (Figures 5 and 6). These excess histones may titrate important replication factors such as Cdc45 or Sld3 (Diffley 2004) and thereby reduce the efficiency of DDK and hence Mcm-helicase function. Thus, loss of Rad53 could reduce DDK and *mcm5-bob1* efficiency, both of which affect Cdc45 function (Zou and Stillman 2000; Sclafani *et al.* 2002). Rad53 may function to remove these inhibitory histones and increase the efficiency of initiation. Therefore, Rad53 kinase may maintain a balance of histones at origins of DNA replication and at replication forks. However, overexpression of Cdc45 or Sld3 has no effect (data not shown).

Alternatively, Rad53 may also affect chromatin structure (Pan *et al.* 2006) at the origin and reduce the efficiency of binding of a number of replication proteins. Probably, histone chaperones (Kaufman *et al.* 1997; Tyler *et al.* 1999; Sharp *et al.* 2001) are not involved in this process as *cac1* and *asf1* also have no effect (Table 4). Furthermore, a reduction in histone expression by *hht2-hhf2*Δ is not phenocopied by a reduction in histone chaperones as *cac1*Δ cannot suppress the effect of *rad53*Δ on both *cdc7* mutant viability and *mcm5-bob1* suppression (data not shown).

In summary, our results support the hypothesis that Rad53 has an important role in the cell cycle that is independent of its checkpoint function, which has been proposed by a number of others (Zhao *et al.* 2001; Gunjan and Verreault 2003; Pan *et al.* 2006). Our results show that this role is most likely the regulation of the initiation of DNA replication because of genetic interactions of Rad53 protein kinase with DDK (Cdc7-Dbf4 kinase) and the MCM helicase, both of which act in replication. Further support for the hypothesis is provided by demonstration of origin binding by Rad53 (Figures 3 and 4) and elevated minichromosome loss in *rad53* mutants (Figure 2).

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