RAD53 Regulates *DBF4* Independently of Checkpoint Function in *Saccharomyces cerevisiae*

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ABSTRACT

The Cdc7p and Dbf4p proteins form an active kinase complex in *Saccharomyces cerevisiae* that is essential for the initiation of DNA replication. A genetic screen for mutations that are lethal in combination with *cdc7-1* led to the isolation of seven *lsd* (*l*ethal with *s*even *d*efect) complementation groups. The *lsd7* complementation group contained two temperature-sensitive *dbf4* alleles. The *lsd1* complementation group contained a new allele of *RAD53*, which was designated *rad53-31*. *RAD53* encodes an essential protein kinase that is required for the activation of DNA damage and DNA replication checkpoint pathways, and that is implicated as a positive regulator of S phase. Unlike other *RAD53* alleles, we demonstrate that the *rad53-31* allele retains an intact checkpoint function. Thus, the checkpoint function and the DNA replication function of *RAD53* can be functionally separated. The activation of DNA replication through *RAD53* most likely occurs through *DBF4*. Two-hybrid analysis indicates that the Rad53p protein binds to Dbf4p. Furthermore, the steady-state level of *DBF4* message and Dbf4p protein is reduced in several *rad53* mutant strains, indicating that *RAD53* positively regulates *DBF4*. These results suggest that two different functions of the cell cycle, initiation of DNA replication and the checkpoint function, can be coordinately regulated through the common intermediate *RAD53*.

THE initiation of DNA replication is a strictly regulated process that is coupled tightly to cell cycle progression and results in the accurate duplication of the genetic material. Orderly cell cycle events ensure that the initiation of DNA replication occurs once, and only once, per cell cycle. At the same time, eukaryotic cells also have evolved mechanisms for reducing or eliminating the result of DNA damage or incomplete replication before completing other cell cycle events, such as mitosis. These surveillance mechanisms, termed checkpoints, ensure that the integrity of the genome is intact before proceeding through crucial cellular events (for reviews see Hartwell and Weinert 1989; Stewart and Enoch 1996; Weinert 1998). Three classes of DNA checkpoints have been described in yeast. One pathway blocks exit from S phase or entry into mitosis if DNA replication is incomplete (Weinert 1992; Allen *et al.* 1994; Weinert *et al.* 1994). The other two classes prevent exit from either G1 or G2 in cells containing damaged DNA (Weinert and Hartwell 1988, 1990; Siede *et al.* 1993). Failure of the cellular restraints imposed normally by a checkpoint can result in genomic instability, increased mutation rates, and ultimately death, if cells continue to divide unchecked.

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Isolation of mutants defective in cell cycle progression led to the identification of *CDC7*, a gene encoding a nuclear serine/threonine kinase that is essential for the initiation of S phase in *Saccharomyces cerevisiae* (Hartwell 1973; Bahman *et al.* 1988; Hollingsworth and Sclafani 1990; Yoon and Campbell 1991). The kinase activity of Cdc7p is required for the initiation of replication (Buck *et al.* 1991; Hollingsworth *et al.* 1992). Also, Cdc7p recently has been shown to be required throughout S phase for origin firing, but not for elongation (Bousset and Diffley 1998; Donaldson *et al.* 1998). Although the level of *CDC7* transcripts appears to be constitutive throughout the cell cycle, Cdc7p kinase activity fluctuates in a cell cycle-dependent manner, peaking at the time S phase begins (Sclafani *et al.* 1988; Jackson *et al.* 1993). This is brought about by association of Cdc7p (Jackson *et al.* 1993) with the product encoded by the *DBF4* gene, whose expression varies in a cell cycle-dependent manner (Chapman and Johnston 1989). Through genetic studies, *CDC7* and *DBF4* have been shown to act at the same point (Kitada *et al.* 1992). Likewise, Cdc7p and Dbf4p have also been shown to interact physically, and both are required to obtain active kinase activity (Jackson *et al.* 1993). The Cdc7p kinase complex is brought to origins of replication via the interaction of Dbf4p with the origin replication complex (ORC), where it is thought to phosphorylate members of the prereplication complex (Dowell *et al.* 1994; Hardy *et al.* 1997). One phosphorylation target is the product of the *MCM2* gene (Lei *et al.* 1997). Phosphorylation of the prereplication complex converts it into an

active replication complex, although exactly how this is achieved is still unknown.

Initiation of the checkpoint program requires the activity of many different proteins. The components of the DNA damage checkpoint machinery generally fall into three classes: sensors, transducers, and targets (Weinert 1998). *RAD9*, *RAD17*, *RAD24*, and *MEC3* are included in the sensor class and are required for response to DNA damage (Lydall and Weinert 1995; Navas *et al.* 1995; Paulovich *et al.* 1997; de la Torre-Ruiz *et al.* 1998). The *POL2*, *RFD5*, and *DPB11* classes are required for the response to the arrest of DNA replication (Araki *et al.* 1995; Navas *et al.* 1995). They are thought to recognize DNA damage or stalled replication forks, and they initiate a protein kinase signal transduction cascade that activates Mec1p and Rad53p (Sanchez *et al.* 1996; Sun *et al.* 1996). The protein kinases Rad53p and Mec1p act as transducers and transmit signals to critical downstream targets (Sanchez *et al.* 1996; Sun *et al.* 1996), leading to the transcription of genes involved in DNA replication and repair (Aboussekhra *et al.* 1996; Kiser and Weinert 1996; Navas *et al.* 1996). Another consequence is the activation of targets that slow or halt the cell cycle and allow the completion of DNA repair or replication (Sidorova and Breeden 1997). So far, only one potential target of this cascade has been identified. Swi6p is modified in a *RAD53*-dependent manner in response to the G1 (DNA damage) checkpoint, which results in the delay of entry into S phase by inhibiting *CLN* transcription (Sidorova and Breeden 1997).

RAD53 (=*MEC2*, *SPK1*, *SAD1*) encodes a dual-specificity protein kinase that is required for all three DNA damage checkpoints at G1, S phase, and G2 (Stern *et al.* 1991; Zheng *et al.* 1993; Allen *et al.* 1994; Weinert *et al.* 1994; Fay *et al.* 1997). It is thought to be part of the transducer class (Weinert 1998). Mutations in *RAD53* have been isolated in screens for checkpoint mutations (Allen *et al.* 1994; Weinert *et al.* 1994), and null mutants of *RAD53* are inviable (Zheng *et al.* 1993; Allen *et al.* 1994; Kim and Weinert 1997). *RAD53* mutants continue to divide unchecked in response to DNA-damaging agents and die as a result (Zheng *et al.* 1993; Allen *et al.* 1994; Weinert *et al.* 1994). Rad53p itself is modified in response to DNA damage in a *MEC1*- and *TEL1*-dependent manner, placing *RAD53* downstream of *MEC1* and *TEL1* (Sanchez *et al.* 1996; Sun *et al.* 1996).

Several lines of circumstantial evidence suggest that in addition to its checkpoint function, *RAD53* could also be involved in the initiation of DNA replication. First, *RAD53* has been shown to be transcriptionally coregulated with DNA synthetic genes (Zheng *et al.* 1993). This regulation is most likely conferred by a pair of consensus *Mlu*I cell cycle box (MCB) regulatory elements within its promoter. These elements are found in the promoters of a number of genes that are transcribed in late G1 and include DNA synthetic enzymes. Second, immunolocalization places Rad53p in the nucleus, where it presumably functions with other DNA replication and repair enzymes (Zheng *et al.* 1993). Third, and probably most compelling, a *RAD53* disruption gives rise to spores that form inviable microcolonies enriched for cells that demonstrate a large budded cell morphology with short mitotic spindles (Zheng *et al.* 1993). This phenotype is consistent with other *cdc* mutants that contain mutations required for DNA synthesis. Because other checkpoint genes can be deleted, it is not entirely clear why *RAD53* is essential. However, it has been postulated that the essential function of *RAD53* could be related to some aspect of DNA replication or the timing of mitosis (Zheng *et al.* 1993; Weinert 1998).

The two different aspects of the cell cycle, initiation of DNA replication and checkpoint function, could be coordinately regulated through DNA metabolism events. As a starting point for our studies, we were interested in identifying genes that act in the same pathway or affect a similar function as *CDC7*. Here we report the isolation of a new allele of *RAD53* in a genetic screen for mutants that are lethal in combination with *cdc7-1*. This allele of *RAD53* retains a fully functional checkpoint. In addition, we show that Rad53p interacts with Dbf4p and controls the level of expression of *DBF4*. Thus, we demonstrate that the checkpoint functions and the DNA replication functions can be functionally separated in *RAD53*. These results suggest strongly that coordination of the two separate functions could occur through a common intermediate, namely, *RAD53*.

MATERIALS AND METHODS

Yeast strains, media, and plasmids: Yeast strains were grown in yeast extract/peptone/dextrose (YEPD) with 2% glucose or in synthetic defined (SD) minimal media supplemented with appropriate amino acids and 2% glucose. All yeast strains used in this study are listed in Table 1. All strains are congenic with A364a, except as marked (Hartwell 1967). Strains PJ69-4a (James *et al.* 1996), Y187, and Y190 (Clontech, Palo Alto, CA) are from a different genetic background and are used for two-hybrid analysis. Standard genetic methods were used for strain construction and tetrad analysis (Sherman *et al.* 1986), and transformation of yeast strains was performed by the lithium acetate method (Ito *et al.* 1983). Disruption of the *PEP4* and *RAD53* genes was done by the one-step gene replacement method (Rothstein 1983). For the *pep4*Δ*::URA3* disruption, plasmid pBR322-*pep4*Δ*::URA3* was digested with *Eco*RI and *Xho*I before transformation. The presence of *pep4*Δ*::URA3* was analyzed using the APE overlay test for CpY activity (Jones 1991). For the *rad53*Δ*::hisG-URA3-hisG* disruption, pPD84 was digested with *Bam*HI before transformation. The presence of the *rad53*Δ*::hisG-URA3-hisG* disruption was analyzed by Southern blot analysis (Ausubel *et al.* 1987). Strains bearing the *rad53*Δ*::hisG-URA3-hisG* disruption were grown on 5-fluoroorotic medium to select for loss of the *URA3* marker, resulting in a strain carrying *rad53*Δ*::hisG* allele.

All plasmids used in this study are listed in Table 2. The *CDC7-ADE3* sectoring plasmids were constructed in several steps. To construct pPD4 (pRS316-*CDC7-ADE3*), a 2.7-kb *Sal*I to *Eco*RI *CDC7* fragment from pRH102 (Hollingsworth *et al.* 1992) was cloned into the *Sal*I to *Eco*RI sites of pRS316, generating pPD1. A 3.5-kb *Bcl*I *ADE3* fragment was then removed

TABLE 1

List of strains used in this study

Strains	Genotype (source)
199	*MAT*α *cdc8-1 can1 gal1 leu2 trp1 ura3*
209	*MAT***a** *cdc7-3 can1 ade1 ade2 gal1 his7 ura3 leu2*
299	*MAT*α *can1 cyh2 gal1 his3 leu2 trp1 ura3*
576	*MAT***a** *cdc7-4 ade2 gal1 ura3*
708	*MAT***a** *cdc7-7 gal1 his3 leu2 trp1 ura3*
P119	*MAT***a** *bob1-1(=mcm5) can1 gal1 his7 leu2 lys2 trp1 ura3*
P257	*MAT*α *bob1-1(=mcm5) ade1 can1 cyh2 gal1 his3 trp1 ura3*
PDY024	*MAT***a** *cdc7-1 ade2 ade3 bar1 cyh2 gal1 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY029	*MAT*α *dbf4-1 ade2 ade3 cyh2 gal1 his7 leu2 lys2 trp1 ura3*
PDY093	*MAT*α *cdc7-1 ade2 ade3 cyh2 gal1 his3 leu2 ura3*
PDY095	*MAT*α *dbf4-1 ade2 ade3 cyh2 gal1 lys2 trp1*
PDY108	PDY024 *lsd1-1 (=rad53-31)*
PDY109	PDY024 *lsd6-1 (=cdc7 null)*
PDY120	PDY024 *lsd7-1 (=dbf4)*
PDY124	PDY024 *lsd2-1*
PDY128	PDY024 *lsd3-1*
PDY129	PDY024 *lsd4-1*
PDY138	PDY024 *lsd5-1*
PDY177	*MAT***a** *cdc7-1 ade2 ade3 gal1 his7 leu2 ura3*
PDY201	*MAT*α *lsd1-1 cdc7-1 ade2 ade3 gal1 his3 his7 leu2 trp1 ura3* + pRS314-*CDC7-ADE3*
PDY206	*MAT*α *lsd1-1 cdc7-1 ade2 ade3 gal1 his7 leu2 ura3* + pRS316-*CDC7-ADE3*
PDY207	*MAT***a** *lsd1-1 cdc7-1 ade2 ade3 gal1 his7 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY208	*MAT*α *lsd2-1 cdc7-1 ade2 ade3 gal1 his7 leu2 ura3* + pRS316-*CDC7-ADE3*
PDY209	*MAT***a** *lsd2-1 cdc7-1 ade2 ade3 gal1 his7 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY210	*MAT*α *lsd3-1 cdc7-1 ade2 ade3 gal1 his7 leu2 ura3* + pRS316-*CDC7-ADE3*
PDY211	*MAT***a** *lsd3-1 cdc7-1 ade2 ade3 gal1 his7 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY212	*MAT*α *lsd4-1 cdc7-1 ade2 ade3 gal1 his7 leu2 ura3* + pRS316-*CDC7-ADE3*
PDY213	*MAT***a** *lsd4-1 cdc7-1 ade2 ade3 gal1 his7 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY214	*MAT*α *lsd5-1 cdc7-1 ade2 ade3 gal1 his7 leu2 ura3* + pRS316-*CDC7-ADE3*
PDY215	*MAT***a** *lsd5-1 cdc7-1 ade2 ade3 gal1 his7 leu2 trp1 ura3* + pRS316-*CDC7-ADE3*
PDY242	*MAT*α *rad53-31 pep4*Δ*::URA3 cdc7-1 ade2 ade3 gal1 his3 his7 leu2 trp1 ura3* + pRS314-*CDC7-ADE3*
PDY258	*MAT*α *rad53-11 pep4*Δ*::URA3 gal1 his3 his7 ura3*
PDY289	*MAT***a** *rad53-31 pep4*Δ*::URA3 ade2 ade3 bar1 gal1 his6 his7 leu2 trp1 ura3*
PDY294	*MAT*α *rad53*Δ*::hisG-URA3-hisG bob1-1 cyh2 gal1 his3 lys2 leu2 trp1 ura3* + pGAP-*RNR1*
PDY305	*MAT*α *can1 cyh2 gal1 his3 leu2 trp1 ura3* + pRS316-7HA-*DBF4*
PDY306	*MAT***a** *rad53-31 cdc7-1 ade2 ade3 gal1 his3 his7 leu2 trp1 ura3* + pRS314-*CDC7-ADE3*, pRS316-7HA-*DBF4*
PDY307	*MAT***a** *rad53-11 bar1 gal1 his6 his7 ura3* + pRS316-7HA-*DBF4*
PDY308	*MAT***a** *rad53*Δ*::hisG bob1-1 can1 cyh2 gal1 leu2 lys2 trp1* + pGAP*RNR1*, pRS316-7HA-*DBF4*
PJ69-4a	*MAT*α *ade2 gal4*Δ *gal80*Δ *his3 lys2 LYS2::GAL1p-HIS3 GAL2p-ADE2 met2::GAL7p-lacZ* (James *et al.* 1996)
TWY310	*MAT*α *rad53-11 ura3 his3 his7* (T. Weinert)
Y187	*MAT*α *ade2 gal4*Δ *gal80*Δ *his3 leu2 met- trp1 ura3 URA3::GAL1*$_{UAS}$*-GAL1*$_{tata}$*-lacZ* (Clontech)
Y190	*MAT***a** *ade2 cyh2 gal4*Δ *gal80*Δ *his3 leu2 lys2 trp1 ura3 LYS2::GAL1*$_{UAS}$*-HIS3*$_{tata}$*-HIS3 URA3::GAL1*$_{UAS}$*-GAL1*$_{tata}$*-lacZ* (Clontech)

from pMW29 (gift from David Stillman) and cloned into the *Bam*HI site of the polylinker of pRS316, generating pPD3. The *ADE3* cassette was then removed from pPD3 and cloned into pPD1 as a *Eco*RI and *Not*I fragment, generating pPD4 (pRS316-*CDC7-ADE3*). The pPD7 (pRS314-*CDC7-ADE3*) plasmid was constructed by removing the 6.2-kb *Not*I to *Sal*I fragment containing both the *CDC7* and *ADE3* genes and ligating it into the *Not*I and *Sal*I sites of the polylinker of pRS314.

The *DBF4* genomic plasmids pPD32 (pRS315-*DBF4*) and pGO117 (pRS425-*DBF4*) were constructed by ligating the genomic 5-kb *Sal*I to *Eco*RI *DBF4* fragment from pDBF4.4 (Chapman and Johnston 1989) into the *Sal*I to *Eco*RI sites of pRS315 and pRS425, respectively.

The *RAD53* genomic plasmids pPD60 (pRS316-*RAD53*) and pPD83 (pRS314-*RAD53*) were constructed by ligating the genomic 5322-bp *Cla*I *RAD53* fragment into the *Cla*I sites of pRS316 and pRS314, respectively.

Plasmid pPD84 (pBS-*rad53*Δ*::hisG-URA3-hisG*) was created in two steps. First, the 5322-bp genomic *RAD53 Cla*I fragment was cloned into the *Cla*I site of pBS KS+ (Stratagene, La Jolla, CA), generating pPD82. This plasmid was then digested with *Sph*I to completion and filled in with Klenow. This removes the *RAD53* promoter and most of the coding region of *RAD53* (nt −477 to +1887). The 3.8-kb *Bam*HI to *Bgl*II *hisG-URA3-hisG* fragment from pNKY51 (Alani *et al.* 1987) was filled in with Klenow and ligated into this site, generating pPD84 (pBS-*rad53*Δ*::hisG-URA3-hisG*).

The *GAL4*$_{DB}$-RAD53 plasmids were constructed as follows. A 1221-bp *Pvu*II fragment of *RAD53* was first cloned into the *Sma*I site in frame of pY2, generating pPD93 [p*GAL4*$_{DB}$*-RAD53i*

TABLE 2

List of plasmids used in this study

Plasmids	Description	Source
pBluescriptKS+	Cloning vector	Stratagene
pRS314	*TRP1* yeast shuttle vector	Sikorski and Hieter (1989)
pRS315	*LEU2* yeast shuttle vector	Sikorski and Hieter (1989)
pRS316	*URA3* yeast shuttle vector	Sikorski and Hieter (1989)
pPD4	pRS316-*CDC7-ADE3* (*URA3*)	This study
pPD7	pRS314-*CDC7-ADE3* (*TRP*)	This study
pPD32	pRS315-*DBF4* (*LEU2*)	This study
pGO117	pRS425-*DBF4* (*LEU2*)	This study
pRS277	p*CDC7* CEN (*LEU2*)	Hollingsworth *et al.* (1992)
pRS288	p*cdc7-1* CEN (*LEU2*)	Hollingsworth *et al.* (1992)
pPD58	YCp50-*RAD53* genomic clone	This study
pPD60	pRS316-*RAD53* (*URA3*)	This study
pPD83	pRS314-*RAD53* (*TRP1*)	This study
pBR322-*pep4*Δ*::URA3*	*pep4*Δ*::URA3* disruption plasmid	Gift from B. Tye
pNKY51	p*hisG::URA3::hisG* cassette	Alani *et al.* (1987)
pPD84	pBS-*rad53*Δ*::hisG-URA3-hisG*	This study
pY2	p$GAL4_{DB}$ (*TRP1*)	Sadowski *et al.* (1992)
pPD93	p$GAL4_{DB}$-*RAD53i* (aa50–aa457) fusion (*TRP1*)	This study
pPD94	p$GAL4_{DB}$-*RAD53* (aa50–aa822) fusion (*TRP1*)	This study
pGO109	p$GAL4_{DB}$-*DBF4* fusion (*TRP1*)	Shellman *et al.* (1998)
pGAD-2F	p$GAL4_{AD}$ (*LEU2*)	Chien *et al.* (1991)
pGO119	p$GAL4_{AD}$-*CDC7* fusion (*LEU2*)	Shellman *et al.* (1998)
pCH441	p$GAL4_{AD}$-*DBF4* fusion (*LEU2*)	Hardy and Pautz (1996)
pGO174	pRS316-7HA-*DBF4* (*URA3*)	This study
pGAP-*RNR1*	p*RNR1* driven off *GAP* promoter (*TRP1*)	T. Weinert

(aa50–aa457)]. pPD94 [p$GAL4_{DB}$-*RAD53* (aa50–aa822)] was constructed by ligating in the 2321-bp *Bam*HI-to-*Hin*dIII fragment of *RAD53* from pPD82 into the *Bam*HI and *Hin*dIII sites of pPD93, reconstructing the 3′ end of *RAD53*. Plasmid pGO174 (pRS316-7HA-*DBF4*) was constructed in several steps. First, the genomic 5-kb *Sal*I to *Hin*dIII *DBF4* fragment from pDBF4.4 (Chapman and Johnston 1989) was cloned into the *Sal*I to *Hin*dIII sites of pRS316, generating pPD2. pPD2 was then digested with *Not*I and filled in with Klenow to eliminate the *Not*I site in the polylinker, generating pGO140. Two PCR fragments were generated from this plasmid template using the following sets of primers and *Pfu* DNA polymerase: T3 primer 5′ ATT AAC CCT CAC TAA AGG GA 3′, HA-*DBF4* primer 5′ TAC AGC GGC CGC ATG CAT AGT CAG GAA CAT CGT ATG GGT ACA TTT TCT TCT TTC TTT TC 3′, *Not*I *DBF4* primer 5′ ATC TGC GGC CGC GTT TCT CCA ACG AAA ATG 3′, and 3′ primer DBF4 5′ ATC GTT GCA TGT GTG AGG 3′. The two PCR fragments were cloned into pGO140 as a three-part ligation: pGO140 plasmid digested with *Sal*I and *Bcl*I, PCR fragment 1 digested with *Sal*I and *Not*I, and PCR fragment 2 digested with *Not*I and *Bcl*I, generating pGO154. Finally, a triple HA tag DNA fragment (Tyers *et al.* 1992) with *Not*I ends was ligated into the *Not*I site of pGO154. The resulting plasmid was shown to contain two contiguous direct repeats of the triple HA tag, resulting in a total of seven HA tags. The 5′ end of the gene was sequenced past the *Bcl*I site to determine whether all HA tags were in frame and no mutations were introduced by PCR amplification. This plasmid can complement the *dbf4-1* temperature-sensitive mutation, and Dbf4p protein can be easily detected by immunoblot analysis using the 12CA5 monoclonal antibody directed against the HA epitope.

Synthetic lethality screen: The synthetic lethality screen was done as described previously (Kranz and Holm 1990; Bender and Pringle 1991; Hardy 1996). Ethyl methanesulfonate (EMS; Sigma, St. Louis, MO) mutagenesis of strain PDY024 was performed under conditions that would generate ~30% survival (Jackson *et al.* 1993). Survival was measured at 28%. A total of 4×10^8 cells was mutagenized and split into 10 different pools. After inactivation of EMS with sodium thiosulfate, cells were washed in 50 mm KH_2PO_4 buffer, pH 7.0, and then were plated at a dilution of 500 colonies per plate on YEPD. The plates were incubated at 23° for 7 days until color had fully developed. Mutagenized cells that demonstrated a rare nonsectored colony morphology were further characterized.

Determination of DNA damage-induced cell cycle delay: For UV survival studies, cells were grown to late-logarithmic phase in liquid culture (10^8 cells/ml), counted, and plated at a dilution of ~500 cfu/plate. Cells were mutagenized by exposure to UV light with a 254-nm source at fluence rate of 0.7 mW/cm^2, as measured with a UVP radiometer for doses of 0, 40, 60, 80, and 100 J/m^2 (Ostroff and Sclafani 1995). The plates were wrapped in aluminum foil to prevent light repair and were incubated at 23° for 7 days and then counted. All experiments were performed in triplicate. Percent survival was determined relative to unirradiated controls.

For hydroxyurea arrest experiments, cells were grown to midlogarithmic phase in liquid YEPD culture (10^7 cells/ml). A small aliquot of cells was removed from each culture as a negative control before adding hydroxyurea. Hydroxyurea was then added to a final concentration of 0.2 m, and aliquots were removed at timed intervals to determine cell number and to score for viability as colony-forming units on YEPD plates (Allen *et al.* 1994). Percentage survival was determined relative to cells that were not exposed to hydroxyurea at the beginning of the experiment.

Two-hybrid assay: Activation domain and DNA-binding domain fusion plasmids were first transformed sequentially into strain PJ69-4a, and selection was carried out on SD-Leu-Trp

plates. To assay for interaction, colonies were plated onto SD-Leu-Trp-Ade plates, and prototrophic growth was analyzed (James *et al.* 1996). For the quantitative β-galactosidase analysis, the DNA-binding constructs were transformed into strain Y190 (Clontech), and the activation domain constructs were transformed into strain Y187 (Clontech). The transformed haploids were mated, and the diploids were selected on SD-Trp-Leu media. β-Galactosidase activity was measured in Miller units, as described previously (Jackson *et al.* 1993; Shellman *et al.* 1998). Three individual colonies from each diploid strain were used to perform the β-galactosidase assays.

Northern blot analysis: A plasmid containing a 7-hemagluttin tag *DBF4* gene under the control of the wild-type *DBF4* promoter was transformed into wild-type, *rad53-31*, *rad53-11*, and *rad53Δ::hisG* strains for Northern, FACS, and immunoblot experiments (see below). The *rad53Δ::hisG* strain was kept alive by overexpression of *RNR1* (Sanchez *et al.* 1996). Northern (RNA) analysis was performed as described previously, with the following exceptions (Dohrmann *et al.* 1992). Total RNA (10 μg) was subjected to electrophoresis through a 1% agarose-formaldehyde gel and was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL). Hybridization probes were generated with a Random Prime DNA labeling system (GIBCO BRL, Gaithersburg, MD) and α-^{32}P (3000 Ci/mmol; New England Nuclear, Boston, MA) from the following templates: for *DBF4*, a 794-bp *Xho*I internal fragment of *DBF4*; for *POL1*, an *Eco*RI 869-bp internal fragment of *POL1*. To detect 18s rRNA, an 18s rRNA DNA primer (5′ GCTTATACTTAGACATGCAT 3′; gift from Judith Jaehning) was labeled with [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. Hybridization and subsequent washes of the Northern blots with the 18s rRNA DNA primer were carried at a temperature of 40°. Autoradiographic bands were quantitated on a Molecular Dynamics PhosphorImager using ImageQuant software.

Fluorescence-activated cell sorter (FACS) analysis: Cells were grown in synthetic defined media at 23°, diluted to 10^6 cells/ml, allowed to grow to a density of 2–4 × 10^6 cells/ml (midlogarithmic phase), and then processed for FACS analysis as described previously (Nash *et al.* 1988). Cell numbers and sizes were determined using a Coulter Mulitsizer II using an aperture tube with a 100-μm orifice and latex beads as size standards.

Immunoblot analysis: For immunoblot analysis, cells were grown in synthetic defined media to midlogarithmic phase (2 × 10^6 cells/ml) and analyzed with a Coulter Multisizer II. A total of 2 × 10^7 cells were processed for yeast extracts. Cells were washed twice in H_2O and then resuspended in 20 μl SDS sample buffer plus protease inhibitors [phenylmethyl sufonyl fluoride (Sigma), 174 ng/ml; leupeptin (Peptide Institute, Inc.), 1.3 ng/ml; and pepstatin (Boehringer Mannheim, Indianapolis, IN), 0.3 ng/ml], and then boiled immediately for 5 min. SDS-PAGE and immunoblot analysis was performed as described previously (Jackson *et al.* 1993), except that the immunoblots were visualized with the ECL enhanced chemiluminescence kit (Amersham). The primary antibodies mouse monoclonal 12CA5 anti-HA (Babco) and rabbit anti-G6PD (Sigma) were used at dilutions of 1:500 and 1:5000, respectively. Secondary horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies (Bio-Rad, Richmond, CA) were both used at a dilution of 1:3000.

RESULTS

***cdc7-1* synthetic lethal screen:** A screen for mutations that are lethal in combination with *cdc7-1* was conducted to identify genes that are in the same genetic pathway or that affect the same biochemical process as *CDC7*. The screen is based on a red/white adenine colony sectoring assay (Kranz and Holm 1990; Bender and Pringle 1991; Hardy 1996). Nonsectored colonies, indicative of mutants that may require the plasmid-borne *CDC7* gene to survive, were designated *lsd* for *l*ethal with *s*even *d*efect. Of the ~15,000 colonies screened, 39 demonstrated a nonsectored red colony phenotype. These 39 strains were backcrossed to PDY093. All the diploids analyzed demonstrated a sectored colony morphology, indicating that the mutations are recessive. Eighteen of these strains were characterized further. The remaining mutants did not demonstrate 2:2 segregation of the sectoring phenotype after meiosis, were unable to mate, or were sporulation defective. The 18 haploid segregants from the original backcross were backcrossed two additional times and sorted into complementation groups by constructing diploids from mutants of opposite mating types. The diploids were tested for their sectoring ability; seven complementation groups were identified this way and designated *lsd1–7*.

We expected to find mutations in *DBF4* on the basis of the fact that *cdc7-1* mutation is known to be synthetically lethal with *dbf4-1* from previous experiments (Kitada *et al.* 1992). Two of the 18 mutants were temperature sensitive and found to contain mutations in *DBF4*. To confirm this conclusion, we showed that the sectoring phenotype was complemented by a *DBF4* plasmid.

The largest complementation group (*lsd6*) contained 11 isolates. Two different types of experiments show that *lsd6* mutants contain null or intragenic mutations in *cdc7*. First, it was noted that 11 diploids, which resulted from a backcross to *cdc7-1* (after loss of pRS316-*CDC7-ADE3-URA3* plasmid), generated two viable and two lethal spores upon tetrad dissection (data not shown). All viable spores from the crosses were *TRP1*, indicating linkage to *CDC7* (<5 cM). Second, a plasmid bearing the *cdc7-1* mutation could complement the *cdc7* null mutation in the chromosome, resulting in a sectored phenotype and supporting our hypothesis. The remaining five complementation groups contained one member each.

Cloning of *LSD1*: Colony sectoring was used to identify plasmids from a YCp50 yeast genomic library (American Type Culture Collection, Rockville, MD) that could complement the *lsd1-1* mutation. Out of ~6900 colonies, one plasmid could complement the defect, and it was designated pPD58. A primer was then used to sequence the regions flanking the insert within the pPD58 library clone. The DNA sequence within the insert was compared to the Saccharomyces Genome Database and found to contain five contiguous open reading frames from chromosome XVI. One of these is *RAD53*, which was found to complement the nonsectoring defect of strain PDY207. This suggests that the *lsd1-1* is an allele of *RAD53*. To prove this, the *PEP4* gene, which is adjacent to *RAD53*, was disrupted with *URA3* in *lsd1-1*. The *lsd1-1* mutation always cosegregated with the *pep4Δ::URA3* marker in subsequent crosses,

indicating that the two genes are tightly linked (<2 cM, data not shown). We conclude that *lsd1-1* encodes an allele of *RAD53*, which we have named *rad53-31*.

A null allele of *RAD53* can be suppressed by *RNR1* in high copy on plasmid pGAP-*RNR1* (Sanchez *et al.* 1996; Desany *et al.* 1998; Zhao *et al.* 1998). However, high-copy *RNR1* cannot suppress the nonsectoring defect of an *lsd1-1* (=*rad53-31*) *cdc7-1* strain, PDY207. This indicates that both the *rad53-31* and *cdc7-1* mutations are required to manifest the synthetic lethal phenotype.

The DNA checkpoint is functional in a *rad53-31* strain: Many alleles of *RAD53* have been isolated in different screens for mutations with a checkpoint defect (Zheng *et al.* 1993; Allen *et al.* 1994; Weinert *et al.* 1994). Cells harboring a *rad53-11* (=*mec2-1*) mutation, for example, exhibit rapid loss of viability in the presence of DNA-damaging agents, such as UV light, because they lack the ability to inhibit cell cycle progression (Zheng *et al.* 1993; Allen *et al.* 1994; Weinert *et al.* 1994). Likewise, *rad53-11* mutants die rapidly in the presence of hydroxyurea, a drug that normally stalls replication forks by limiting nucleotide availability through inhibition of ribonucleotide reductase (Allen *et al.* 1994; Weinert *et al.* 1994). We therefore tested *rad53-31* for a checkpoint defect. As expected, the *rad53-11* strain showed a marked decline in viability in response to increasing UV doses (Figure 1A). Surprisingly, the *rad53-31* strain exhibited a sensitivity to UV damage comparable to that of the wild-type strain control (Figure 1A).

For analysis of the S phase checkpoint, survival of wild-type, *rad53-11*, and *rad53-31* strains in the presence of 200 mm hydroxyurea was examined. Again, as expected, the *rad53-11* strain demonstrated a marked decline in viability (Figure 1B). In contrast, the *rad53-31* strain, like the wild-type, demonstrated no significant loss in viability when grown in the presence of hydroxyurea. We conclude that *rad53-31* is still proficient in checkpoint function.

Genetic interactions among *RAD53* alleles and several cell division cycle mutations: A *cdc7 rad53-11* (=*mec2-1*) double mutant is viable (Weinert *et al.* 1994). This presents an apparent contradiction to our identification of *rad53-31* as being lethal in combination with *cdc7-1*. However, we used a different *cdc7* allele for our studies. To resolve this apparent discrepancy, we crossed *rad53-11* (=*mec2-1*) to all of the currently available *cdc7* mutants in our laboratory, and we assayed tetrads for their ability to form *rad53-11 cdc7-x* spores (Table 3A). Three alleles of *CDC7* demonstrated a synthetic effect in combination with *rad53-11* at permissive temperature. Double mutants could not be recovered for *rad53-11* and *cdc7-3* or *cdc7-7*, and the combination of *rad53-11* with *cdc7-1* exhibited a slow-growth phenotype. We confirmed the previous report that *cdc7-4 rad53-11* double mutants are viable (Weinert *et al.* 1994). We conclude *RAD53* demonstrates a genetic interaction with *CDC7*.

The *rad53-11* (=*mec2-1*) is synthetically lethal with *cdc8-1* (Weinert *et al.* 1994). Crosses of *cdc8-1* with *rad53-31* found that *cdc8-1 rad53-31* double mutants could be easily recovered (Table 3B). We conclude from this experiment that the *cdc8-1 rad53-11* lethal interaction must be the result of the loss-of-checkpoint function in *rad53-11* (Weinert *et al.* 1994). We believe that *cdc8-1 rad53-31* double mutants are viable because *rad53-31* retains an intact checkpoint function.

We reasoned that, because *rad53-31* was lethal with

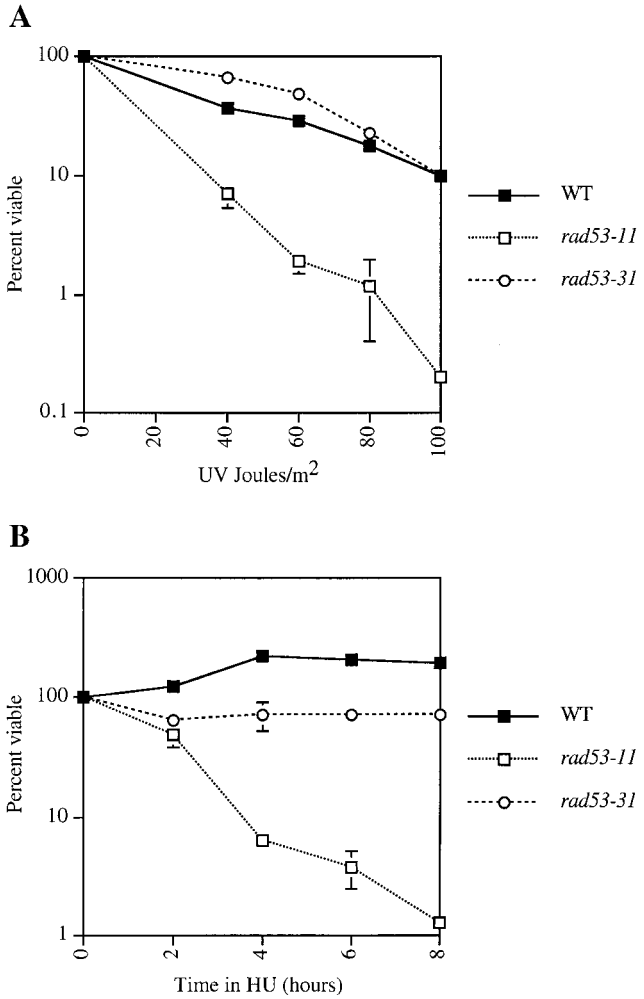

Figure 1.—Absence of checkpoint deficiency in the *rad53-31* strain. (A) UV sensitivity of wild-type and *rad53* mutant strains. Wild-type strain 299 (solid square) and the *rad53* mutant strains TWY310, *rad53-11* (open square) and PDY206, *rad53-31* (open circle) were grown to late logarithmic phase in liquid culture, plated onto YEPD plates at an appropriate dilution (200–500 cfu/plate), irradiated with the indicated doses of UV light, and percent survival was determined relative to unirradiated controls. Shown are the averages of three-independent plates per dose of UV light. (B) Hydroxyurea sensitivity of wild-type and *rad53* mutant strains. Wild-type strain 299 (solid square) and *rad53* mutant strains TWY310, *rad53-11* (open square) and PDY206, *rad53-31* (open circle) were grown to midlogarithmic phase in liquid culture, and hydroxyurea was added (0.2 m). Aliquots were removed at timed intervals to determine cell number and to score for viability as colony forming units on YEPD plates. Percentage of survival was determined relative to cells just before adding hydroxyurea. Three independent plates were scored for each individual time point.

TABLE 3

Genetic interactions between *rad53* and several cell division cycle mutations

Strain	X*rad53-11* (PDY258)
A. Interaction between *rad53-11* and various *cdc7* alleles	
Wild type (299)	Viable (10 tetrads)
cdc7-1 (PDY177)	Slow growth phenotype (18 tetrads)
cdc7-3 (209)	Synthetic lethality/inviable (12 tetrads)
cdc7-4 (576)	Viable (12 tetrads)
cdc7-7 (708)	Synthetic lethality/inviable (33 tetrads)
Strain	X*rad53-31* (PDY289)
B. Interaction between *rad53-31* and *cdc8-1*	
cdc8-1 (199)	Viable (18 tetrads)
C. Interaction between *rad53-31* and *dbf4-1*	
dbf4-1 (PDY029)	Slow growth phenotype (31, 24 tetrads[a])

Genetic interactions between *rad53* and various cell cycle mutations were tested by crossing individual strains together. The diploids were sporulated and dissected and the resulting segregants were grown at the permissive temperature of 23°. The *rad53-11* and the *rad53-31* alleles were marked by a *pep4*Δ*::URA3* marker, which is located directly adjacent to the *RAD53* gene. The number in parentheses indicates how many informative tetrads were dissected for each cross (except in the case of the *dbf4-1* cross, see below for details). In crosses that demonstrated a synthetic lethality/inviable phenotype, no viable double mutants were detected. In addition, no significant deviation from the 1PD:4T:1NPD ratio was observed for all the crosses, as predicted for the segregation of two unlinked genes (except for *dbf4-1*, see below). An extremely slow growth phenotype was detected in tetrad segregants from the *cdc7-1* cross. Upon restreaking, double mutants from the *cdc7-1* cross grew very poorly.

[a] For the *dbf4-1* cross, double mutants could be obtained; however, results were complicated by the fact that out of the original 31 tetrads dissected, the *dbf4-1* mutation reverted to wild type in 22 cases, generating many 3:1 and 4:0 segregation patterns for temperature resistance. To prove that double mutants were viable, this diploid was then transformed with the pRS425-*DBF4* to cover the *dbf4-1* mutation, 24 tetrads were dissected, and plasmids were allowed to be lost from the tetrad segregants. In this case, *dbf4-1 rad53-31* double mutants were easily obtained, and *dbf4-1* always segregated 2:2.

cdc7-1, it might also be lethal with *dbf4-1* because *CDC7* and *DBF4* act at the same point in the pathway (Kitada *et al.* 1992). The *rad53-31 dbf4-1* double mutant could be recovered if the *dbf4-1* mutation in the diploid was first complemented by wild-type *DBF4* on a plasmid, and then the plasmid was allowed to be lost in tetrad segregants (Table 3C). The *rad53-31 dbf4-1* double mutant demonstrated a synthetic growth defect. We conclude that *RAD53* demonstrates a genetic interaction with *DBF4*.

The *bob1-1* mutation cannot suppress a *rad53*Δ*::URA3* null allele: A recessive mutation in *BOB1*(*MCM5*/*CDC46*) bypasses the requirement for the essential S phase activators Cdc7p and Dbf4p at the G1/S boundary (Jackson *et al.* 1993; Hardy *et al.* 1997). Given the fact that *RAD53* interacts genetically with *CDC7* and *DBF4* (see above), we asked if *bob1-1* could bypass the requirement for the *RAD53* gene. A *rad53*Δ*::URA3* disruption was constructed in a heterozygous diploid strain carrying a *bob1-1*, analyzed by Southern blot to confirm the disruption (data not shown), allowed to sporulate, and individual spores were analyzed for viability (Table 4A). The results are complicated by the fact that strains bearing *rad53*Δ*::URA3* frequently generate second site suppressors (Zheng *et al.* 1993; Kim and Weiner t 1997; Desany *et al.* 1998). The number of 3:1 and 4:0 viable:lethal tetrad patterns suggests that *bob1-1* is not responsible for the suppression (Table 4A). This was confirmed by performing a second cross where *bob1-1* was homozygous in the diploid. Many 3:1 and 2:2 viable:lethal segregation patterns were generated, indicating that a second site suppressor was responsible for the suppression, not *bob1-1* (Table 4B). We conclude that *bob1-1* is unable to bypass *rad53*Δ*::URA3*.

Interaction of Rad53p and Dbf4p: Given that *RAD53* interacts genetically with *CDC7* and *DBF4*, we asked if Rad53p could interact physically with Cdc7p and/or Dbf4p, as assayed by the two-hybrid method. The *GAL4*-DB-*RAD53* (aa50–aa822) could complement *rad53*Δ*::URA3*, indicating that it encodes a functional protein (data not shown). The *GAL4*-DB *RAD53i* (aa50–aa457), which lacks C-terminal sequences, failed to complement the *rad53*Δ*::URA3* (data not shown). The results show that Rad53p interacts weakly with Dbf4p, but not with Cdc7p (Figure 2). In addition, the Rad53p fusion construct that lacks the C-terminal sequences failed to interact with either Cdc7p or Dbf4p, suggesting that the C-terminal sequences are necessary for interaction with Dbf4p. Previously described interactions were seen between Dbf4p and Cdc7p, and between Dbf4p and Dbf4p (Jackson *et al.* 1993; Shellman *et al.* 1998).

Several attempts were made to show that Dbf4p and Rad53p interact directly using other physical methods. Rad53p/Dbf4p coimmunoprecipitation and GST-Rad 53p/Dbf4p interaction experiments failed to detect an interaction (data not shown). This may reflect a transient or weak interaction between Rad53p and Dbf4p that can only be detected by the sensitive two-hybrid interaction assay. Nevertheless, two independent two-hybrid assays could detect a significant interaction between Rad53p and Dbf4p.

***RAD53* regulates *DBF4* expression at the mRNA and protein levels:** To understand how *rad53-31* manifests its synthetic lethal effect with *cdc7-1*, we asked whether *DBF4* mRNA and/or Dbf4p protein levels were altered in various *RAD53* mutants. The level of *DBF4* message was significantly reduced in the *rad53*Δ*::hisG* strain, exhibiting about fivefold less mRNA than the wild type (Figure 3A). This was not a result of overexpression of the *RNR1* gene because wild-type cells that overexpress *RNR1* demonstrate no difference in *DBF4* expression (data not shown). In addition, the Northern blot results demonstrated that the level of *DBF4* message in the *rad53-11* strain was reduced about twofold. Surprisingly,

TABLE 4

The *bob1-1*(*mcm5*) mutation does not suppress a null mutation in *rad53*Δ*::URA3*

Relevant genotype of strain dissected	Type of tetrad	Number
A. *bob1-1* or second site suppressor suppresses *rad53*Δ*::URA3* mutation		
bob1/+ +/*rad53*Δ*::URA3*	4:0 viable:inviable	0
(299 × P119)	3:1 viable:inviable	9[a]
	2:2 viable:inviable	16
	Total tetrads dissected	25
B. Backcross to determine if viable segregants are suppressed by *bob1-1* or by a second site suppressor		
bob1/bob1 +/*rad53*Δ*::URA3 sup*?/+	4:0 viable:inviable	13
	3:1 viable:inviable	7
	2:2 viable:inviable	4
	Total tetrads dissected	24

[a] Viable *bob1-1 rad53*Δ*::URA3* from part A was backcrossed to *bob1-1* strain.

the level of *DBF4* message was increased in the *rad53-31* strain.

DBF4 is an MCB box-regulated gene that is expressed just before S phase (Lowndes *et al.* 1992; Zheng *et al.* 1993). Thus, it was possible that *RAD53* could be acting on the expression of this class of genes. If so, one would expect another MCB box-regulated gene, such as *POL1*, to be downregulated in the same manner in the *rad53*Δ*::hisG* strain. However, the *POL1* mRNA level was not significantly different between wild-type and *rad53*Δ*::hisG* strains (Figure 3A). We conclude that *RAD53* does not universally affect MCB box-regulated genes.

We hypothesized that the elevated levels of *DBF4* (and *POL1*) message in the *rad53-31* strain could be a result of cells being shifted toward one phase of the cell cycle. To test this possibility, FACS analysis was performed on the yeast strains that were used to make the extracts (Figure 3C). Most of the *rad53-31* cells were in the G2 phase of the cell cycle. It should be noted that this is the first phenotypic defect we have observed for strains that bear only the *rad53-31* genotype. The level of *DBF4* message was then compared between wild-type and *rad53-31* mutant extracts that were prepared from cells arrested at the same stage of the cell cycle (Figure 3B). Cells were grown first to midlogarithmic phase and then arrested in G1/S in the presence of 200 mm hydroxyurea for 4 hr. *DBF4* message is only moderately reduced in the *rad53-31* strain (Figure 3B).

To test the level of Dbf4p protein expression in the four strains, cells carrying a plasmid with a HA-tagged *DBF4* plasmid were grown to midlogarithmic phase and then harvested to prepare the cells for FACS and the extracts for immunoblot analysis (Figure 4). FACS profiles generated for the four strains were similar to those shown in Figure 3. Immunoblots were then probed with antibodies against 7HA-DBF4p and G6PDp. Dbf4p was undetectable in the *rad53*Δ*::hisG* strain, indicating that *RAD53* positively regulates Dbf4p. Longer exposures revealed a very low level of Dbf4p expression in the *rad53*Δ*::hisG* strain (data not shown). The level of Dbf4p protein expression in the *rad53-31* and *rad53-11* strains was similar to that seen for mRNA expression. Dbf4p protein levels were reduced in the *rad53-11* strain, whereas they were slightly increased in the *rad53-31* strain. We conclude from these experiments that *DBF4* is regulated in a *RAD53*-dependent manner at both the mRNA and protein levels.

DISCUSSION

The activation of DNA repair mechanisms and simultaneous activation of cell cycle arrest by DNA damage checkpoints results in minimizing the effects of DNA damage to eukaryotic cells. Failure of the cellular restraints imposed normally by a checkpoint can result in increased mutation rates, genomic instability, and ultimately death if cells continue to divide unchecked. It is perhaps not so surprising that the signals involved in eukaryotic DNA checkpoint control could be integrated with other DNA replication functions. It has been suggested that the *RAD53* gene, which is involved in the DNA checkpoint, encodes a signal-transducing kinase that could integrate several of these functions (Zheng *et al.* 1993; Weinert 1998).

It has been postulated that *RAD53* encodes dual functions, including positive regulation of replication and negative regulation of cell cycle progression (Sun *et al.* 1996). Teleologically, a cell needs to inhibit cell cycle progression for the DNA repair, but replication enzymes need to be activated to repair damage. This predicts that the DNA replication and the checkpoint functions could be separable.

To this point, the evidence is circumstantial regarding the role of *RAD53* in the control of DNA replication. Several lines of evidence suggest that *RAD53* is associated with DNA replication: transcriptional coregulation with other DNA synthetic enzymes, the terminal arrest phenotype or *rad53*Δ null mutants, the nuclear localization of Rad53p, and the essential function of *RAD53* (Zheng *et al.* 1993). Weinert (1998) suggested that the

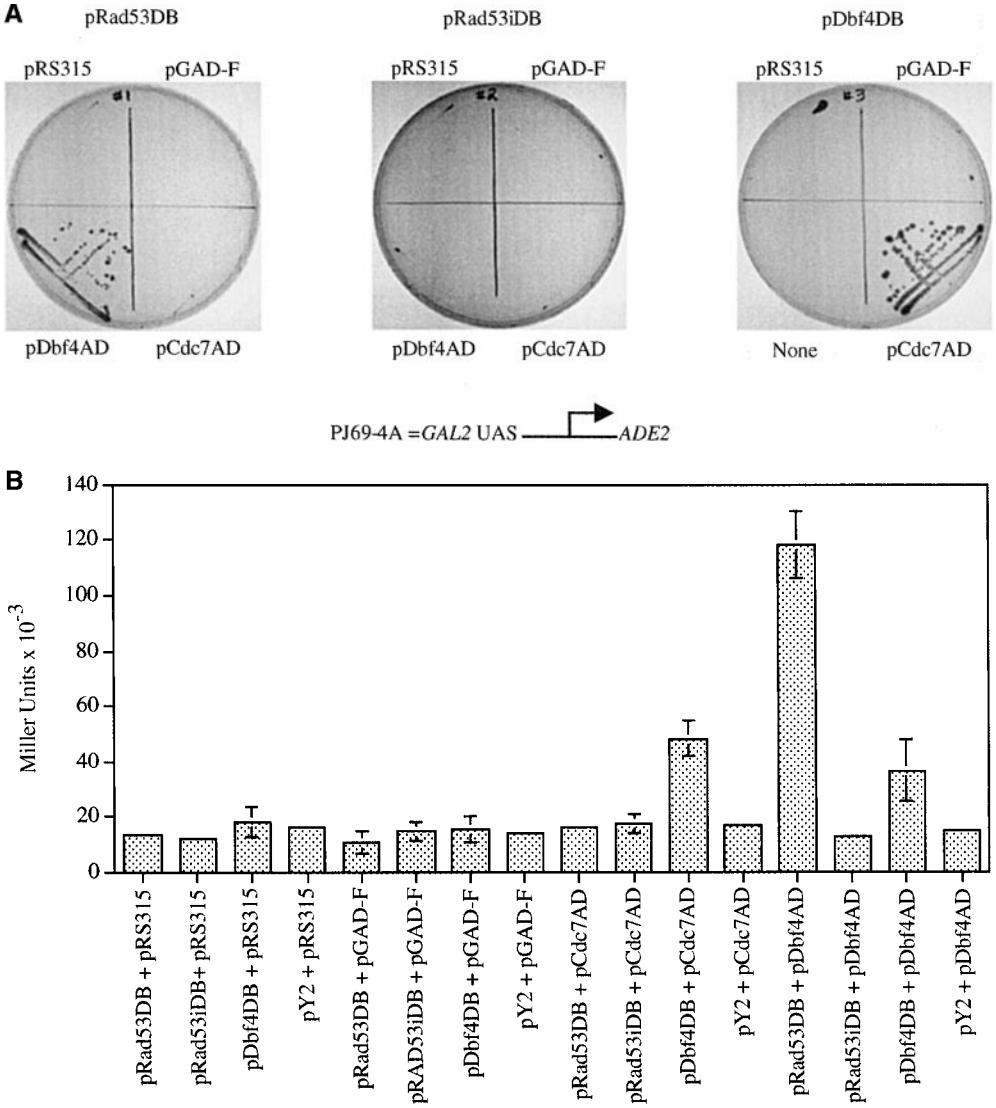

Figure 2.—Interaction of Rad53p with Dbf4p. (A) Prototrophic growth in strain PJ69-4a is dependent on the interaction between *RAD53* and *DBF4* two-hybrid fusions. Strain PJ69-4a, which contains *ADE2* under *GAL2* promoter control, was transformed with the indicated two-hybrid fusion plasmids, and selection was executed on SD-Leu-Trp plates. To assay for interaction, colonies were streaked onto SD-Leu-Trp-Ade plates, shown above. pRad53DB fuses aa50–aa822 to the Gal4pDNA-binding domain, whereas pRad53i fuses aa50–aa457. (B) Quantitative liquid β-galactosidase assays. The indicated DNA-binding domain and activation domain plasmids were transformed into the strains Y187 and Y190, strains were mated, and quantitative β-galactosidase measurements were made from diploid yeast extracts. Assays were completed for three independent colonies from each diploid strain.

essential function of *RAD53* could be related to the timing of mitosis or some aspect of DNA replication.

The experiments performed here provided the strongest evidence yet that there is a direct requirement for *RAD53* in the initiation of DNA replication. Our results indicate that the checkpoint function of the *rad53-31* allele remains intact. At the same time, the *rad53-31* allele is lethal in combination with *cdc7-1*, a gene that is intimately related to the initiation of DNA synthesis. Indeed, it appears that the checkpoint and replication functions of *RAD53* can be separated. This effect can only be seen in combination with *cdc7-1* because mutations that completely knock out the replication function of *RAD53* presumably also knock out the checkpoint function and perhaps the essential function of *RAD53*. Similarly, a synthetic growth defect was seen by *rad53-31* in combination with *dbf4-1*. The *RAD53* synthetic defects seen in combination with both *CDC7* and *DBF4* imply that *RAD53* affects a similar biochemical process or a similar function as *CDC7* and *DBF4*. Another possibility is that *RAD53* acts in the same genetic pathway as *CDC7* and *DBF4*. The allele specificity seen with *rad53-11* and different *cdc7* alleles illustrates the fact that a certain level of Cdc7p kinase activity is required in combination with *rad53-11*, or the cells become inviable. As measured originally through segregation lag experiments, a hierarchy of function of *cdc7* mutants at permissive temperature was determined (Hollingsworth *et al.* 1992). It is clear that *cdc7-3* and *cdc7-7* have the least activity at permissive temperature, whereas the *cdc7-4* has the highest level of activity. This explains why *cdc7-4 rad53-11* (=*mec2-1*) double mutants were originally found to be viable (Weinert *et al.* 1994). The level of activity of *cdc7* mutant alleles most likely determines the allele-specific interaction with *rad53-11*. In addition, when analyzed by FACS, the *rad53-31* allele demonstrates a G2 profile that is reminiscent of defects in other DNA replication genes, such as in *orc5-1* and *mcm2* mutants (Yan *et al.* 1991; Loo *et al.* 1995). Finally, Rad 53p was shown to interact with Dbf4p in two-hybrid ex-

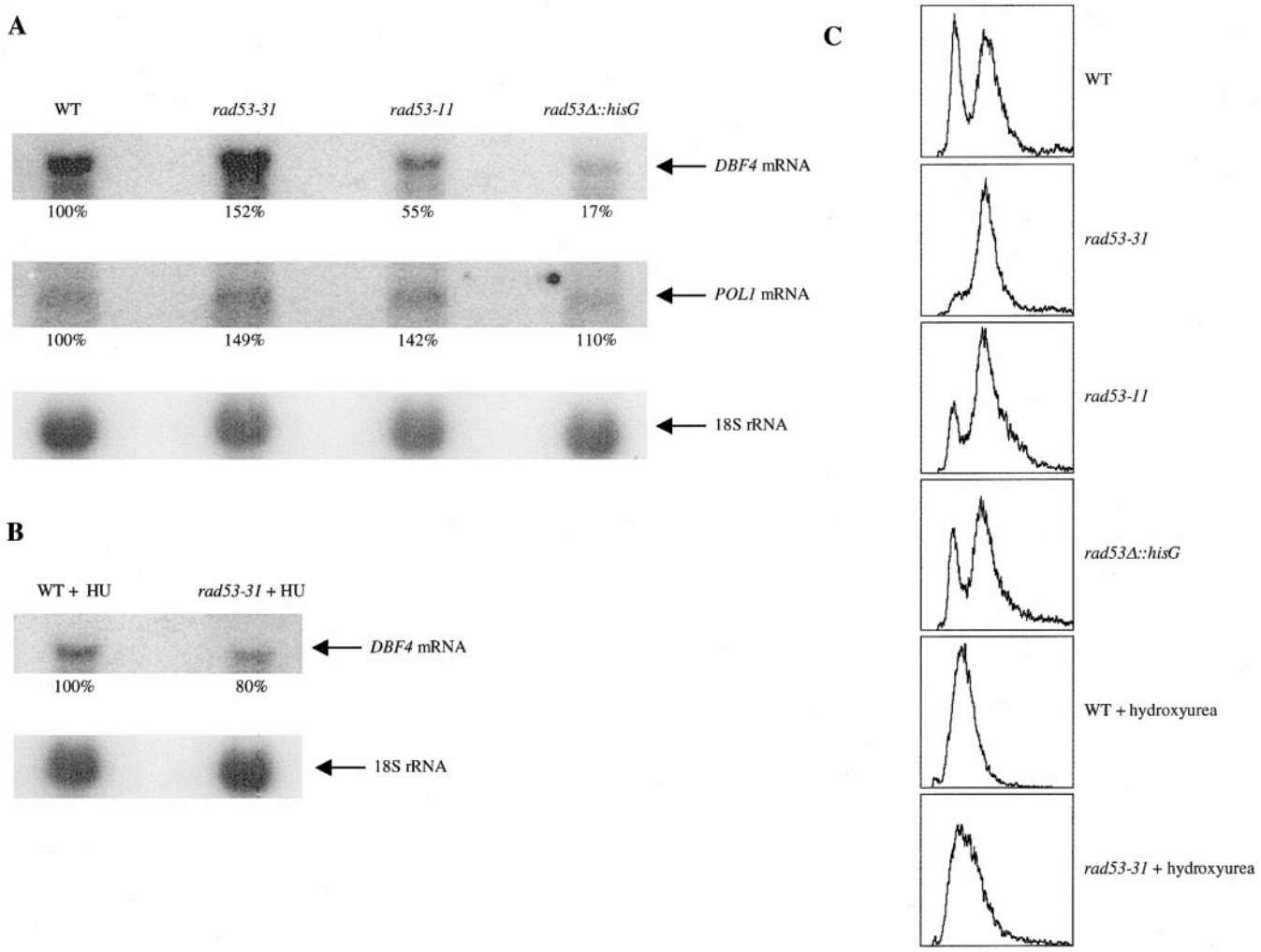

Figure 3.—Steady-state level of *DBF4* mRNA is reduced in the *rad53Δ::hisG* strain. (A) A Northern blot was analyzed containing RNA prepared from four isogenic strains: wild-type strain PDY305, *rad53-31* strain PDY306, *rad53-11* strain PDY307, and *rad53Δ::hisG* strain PDY308. The Northern blot was probed sequentially with a *DBF4* probe, a *POL1* probe, and then a probe against 18s rRNA for an internal control. Cells were grown to midlogarithmic phase before harvesting for RNA analysis and FACS (see C). Autoradiographic bands were quantitated on a Molecular Dynamics PhosphorImager, normalized to the 18s rRNA internal control, and compared to wild type, which was set at 100%. (B) *rad53-31* cells blocked in G1/S demonstrate moderately reduced levels of *DBF4* message. Cells were grown to midlogarithmic phase and cultured for 4 hr in the presence of 0.2 m hydroxyurea, and were then harvested for RNA analysis and FACS. (C) FACS analysis of isogenic yeast strains used to prepare RNA extracts.

periments. Taken together, the genetic and two-hybrid studies strongly suggest that *RAD53* has a positive role in regulating DNA replication.

The predominant G2 population seen in the *rad53-31* mutant via FACS analysis could also be a consequence of the *rad53-31* allele generating a weak but constitutive checkpoint signal. Perhaps the synthetic lethality seen with the *cdc7-1 rad53-31* double mutant is a combination of reduced activity of the *cdc7-1* gene product and a dominant but weak checkpoint signal from *rad53-31*.

Given the fact that the *bob1-1* mutation can bypass the requirement for *CDC7* and *DBF4* (Jackson *et al.* 1993; Hardy *et al.* 1997), why is it that *bob1-1* cannot suppress *rad53Δ::URA3*? It is probable that *bob1-1* cannot bypass the checkpoint function absent in *rad53Δ::URA3* because it is required for integrating the G1, S, and G2 checkpoints. Perhaps the cell cannot survive without all three functions. Alternatively, *RAD53* may be required for other essential functions, such as expression of *RNR1* (Desany *et al.* 1998).

The two lines of evidence presented here also illustrate that *RAD53* exerts its positive control through *DBF4*. First, two-hybrid studies suggest that Rad53p may interact directly through Dbf4p. Second, *RAD53* regulates the expression of *DBF4* at the message and protein levels. The *DBF4* message is reduced fivefold, and the levels of Dbf4p protein are virtually undetectable in a *rad53Δ::hisG* strain. This indicates that *RAD53* regulates *DBF4* positively. At this point, we do not have an explanation for why either *DBF4* message or Dbf4p protein is

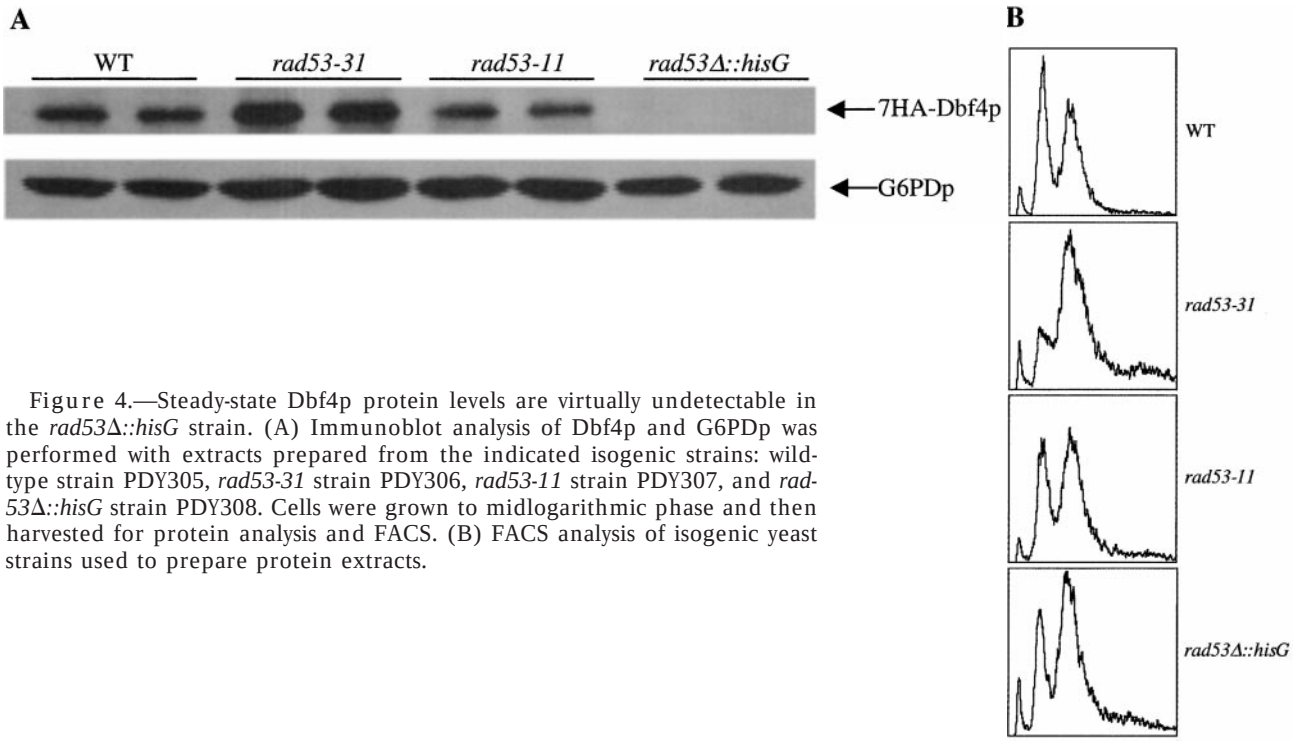

Figure 4.—Steady-state Dbf4p protein levels are virtually undetectable in the *rad53Δ::hisG* strain. (A) Immunoblot analysis of Dbf4p and G6PDp was performed with extracts prepared from the indicated isogenic strains: wild-type strain PDY305, *rad53-31* strain PDY306, *rad53-11* strain PDY307, and *rad-53Δ::hisG* strain PDY308. Cells were grown to midlogarithmic phase and then harvested for protein analysis and FACS. (B) FACS analysis of isogenic yeast strains used to prepare protein extracts.

slightly increased in the *rad53-31* strain. Could a quantitative difference in the Dbf4p protein be responsible for generating the synthetic lethal defect of a *rad53-31 cdc7-1* strain? We know that lowering the level of *DBF4* message twofold in a *cdc7-1* background is still viable (P. Dohrmann, unpublished results). This suggests that the reason *rad53-31* is lethal with *cdc7-1* is not quantitative because the level of *DBF4* message seen in a *rad53-31* strain is 80% of wild type (Figure 3B). Therefore, it follows that *RAD53* must regulate some qualitative difference in Dbf4p protein.

We would hypothesize that perhaps Rad53p binds to and modifies Dbf4p protein. The qualitative difference in Dbf4p activity would be partly responsible for the activation of the DNA replication initiation program. In addition, the activation of *DBF4* transcription and/or degradation of *DBF4* message through *RAD53* could be through a positive feedback loop based on the level of activity of the Dbf4p protein. This would explain reasonably why the *DBF4* message and protein levels in *rad53-31* mutants are not affected severely. At the same time, in *rad53Δ::hisG* mutants, Rad53p is unavailable to modify Dbf4p, and as a consequence, *DBF4* message and Dbf4p protein rapidly disappear.

With the dramatic reduction of Dbf4p protein, the cells are still viable in the *rad53Δ::hisG* strain. Perhaps the essential role of *RAD53* is to regulate the expression of *RNR1* because high-copy expression of *RNR1* can bypass the *rad53Δ::hisG* defect. At the same time, *RAD53* may also regulate *DBF4* levels to ensure rapid S phase entry through this positive feedback loop. Future experiments will address whether Cdc7p kinase activities are altered in various *RAD53* mutant strains. This may help elucidate the role of *RAD53* in controlling Dbf4p activity.

In conclusion, we have demonstrated that the multiple functions of *RAD53* can be separated. These results suggest strongly that *RAD53* is a common intermediate between a checkpoint and DNA replication function.

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