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 (lethal with seven defect) 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.

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 (Bousslet and Diffl ey 1998; Donal dson 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 (Scl afani 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 John ston 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 DBP11 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 (Sidoro va 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 MluI 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 othercdc 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

Yeasts strains, media, and plasmids: Yeast strains were grown in yeast extract/peptone/dextrose (YPED) 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 (Y) 69-4a (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 PEF4 and RAD53 genes was done by the one-step gene replacement method (Rot hrst ein 1983). For the ppp4::URA3 disruption, plasmid pBR322::pdp4::URA3 was digested with EcoRI and Xhol before transformation. The presence of ppp4::URA3 was analyzed using the APE overlay test for CpY activity (Jones 1991). For the rad53::hisG::URA3::hisG disruption, pBD4 was digested with BamHI before transformation. The presence of rad53::hisG::URA3::hisG disruption was analyzed by Southern blot analysis (Ausubel et al. 1987). Strains bearing the rad53::hisG::URA3::hisG disruption were grown on 5-fluoroorotic medium to select for loss of the URA3 marker, resulting in a strain carrying rad53::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 SalI to EcoRI CDC7 fragment from pRH102 (Hollingsworth et al. 1992) was cloned into the SalI to EcoRI sites of pRS316, generating pDP1. A 3.5-kb BglI ADE3 fragment was then removed
from pMW29 (gift from David Stillman) and cloned into the BamHI site of the polylinker of pRS316, generating pPD3. The ADE3 cassette was then removed from pPD3 and cloned into pPD1 as a EcoRI and NotI fragment, generating pPD4 (pRS316-CDC7-ADE3). The pPD7 (pRS314-CDC7-ADE3) plasmid was constructed by removing the 6.2-kb NotI to SalI fragment containing both the CDC7 and ADE3 genes and ligating it into the NotI and SalI 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 SalI to EcoRI DBF4 fragment from pDBF4.4 (Chapman and Johnston 1989) into the SalI to EcoRI 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 ClaI RAD53 fragment into the ClaI sites of pRS316 and pRS314, respectively. Plasmid pPD84 (pBS-rad53Δ::hisG-URA3-hisG) was created in two steps. First, the 5322-bp genomic RAD53 Claf fragment was cloned into the ClaI site of pBS KS+ (Stratagene, La Jolla, CA), generating pPD82. This plasmid was then digested with SphI 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 BamHI to BglII hisG-URA3-hisG fragment from pNK51 (Alani et al. 1987) was filled in with Klenow and ligated into this site, generating pPD84 (pBS-rad53Δ::hisG-URA3-hisG).

The GAL4υ-RAD53 plasmids were constructed as follows. A 1221-bp PvuII fragment of RAD53 was first cloned into the SmaI site in frame of pY2, generating pPD93 [pGAL4υ-RAD53]
TABLE 2
List of plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pBluescriptKS+</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
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<td>pRS314</td>
<td>TRP1 yeast shuttle vector</td>
<td>Sikorski and Hiet er</td>
</tr>
<tr>
<td>pRS315</td>
<td>LEU2 yeast shuttle vector</td>
<td>Sikorski and Hiet er</td>
</tr>
<tr>
<td>pRS316</td>
<td>URA3 yeast shuttle vector</td>
<td>Sikorski and Hiet er</td>
</tr>
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<td>pPD4</td>
<td>pRS316·Cyc7·ADE3 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>pPD7</td>
<td>pRS314·Cyc7·ADE3 (TRP)</td>
<td>This study</td>
</tr>
<tr>
<td>pPD32</td>
<td>pRS315·DBF4 (LEU2)</td>
<td>This study</td>
</tr>
<tr>
<td>pGO117</td>
<td>pRS425·DBF4 (LEU2)</td>
<td>This study</td>
</tr>
<tr>
<td>pRS277</td>
<td>pDC7 CEN (LEU2)</td>
<td>Hollingsworth et al.</td>
</tr>
<tr>
<td>pRS288</td>
<td>pdc7·1 CEN (LEU2)</td>
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<tr>
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<td>Ycp50·RAD53 genomic clone</td>
<td>This study</td>
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<td>pPD60</td>
<td>pRS316·RAD53 (URA3)</td>
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</tr>
<tr>
<td>pPD83</td>
<td>pRS314·RAD53 (TRP1)</td>
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<td>pBR322·pep4Δ·URA3</td>
<td>pepΔ·URA3 disruption plasmid</td>
<td>Gift from B. Tye</td>
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<td>pBS·rad53·:hisG-URA3·hisG</td>
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<td>pGAL4 (TRP1)</td>
<td>Sadowski et al.</td>
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<tr>
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<td>This study</td>
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<tr>
<td>pPD94</td>
<td>pGAL4·Rad53 (aa50·aa822) fusion (TRP1)</td>
<td>This study</td>
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<td>pGO109</td>
<td>pGAL4·DBF4·fusion (LEU2)</td>
<td>Shellman et al.</td>
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<td>pGAL4 (LEU2)</td>
<td>Chien et al.</td>
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<td>Shellman et al.</td>
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<td>pCH441</td>
<td>pGAL4·DBF4·fusion (LEU2)</td>
<td>Hardy and Pautz</td>
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<td>pGO174</td>
<td>pRS316·7HA·DBF4 (URA3)</td>
<td>This study</td>
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<tr>
<td>pGAP·RNR1</td>
<td>RNR1 driven off GAP promoter (TRP1)</td>
<td>T. Weinert</td>
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</table>

(aa50·aa457). pPD94 [pGAL4·Rad53 (aa50·aa822)] was constructed by ligation in the 2321-bp BamHI-to-HindIII fragment of RAD53 from pPD82 into the BamHI and HindIII sites of pPD93, reconstructing the 3’ end of RAD53. Plasmid pGO174 (pRS316·6H·DBF4) was constructed in several steps. First, the genomic 5-kb SalI to HindIII DBF4 fragment from pDBF4.4 (Chapman and Joh n st on 1989) was cloned into the SalI to HindIII sites of pRS316, generating pPD2. pPD2 was then digested with NotI and filled in with Klenow to eliminate 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 fluence rate of 0.7 mJ/cm^2, as measured with a UVP radiometer for doses of 0, 40, 60, 80, and 100 J/m^2 (Ostroff and Sci a fani 1995). The plates were wrapped in aluminum foil to prevent light repair and were incubated at 23°C 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 mid-logarithmic phase in liquid YEPD culture (10^5 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 (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 x 10^6 cells was mutagenized and split into 10 different pools. After inactivation of EMS with sodium thiosulfate, cells were washed in 50 mm KH2PO4 buffer, pH 7.0, and then were plated at a dilution of 500 colonies per plate on YEPD. The plates were incubated at 23°C for 7 days until color had fully developed. Mutagenized cells that demonstrated a rare nonsectored colony morphology were further characterized.
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; Shelnman 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-hemaglutinin tag DBF4 gene under the control of the wild-type DBF4 promoter was transformed into wild-type, rad53-31, rad53-11, and rad53::hisG strains for Northern, FACS, and immunoblot experiments (see below). The rad53::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-32P (3000 Ci/mmol; New England Nuclear, Boston, MA) from [γ-32P]ATP (6000 Ci/mmol) and T4 poly-nucleotide kinase. Hybridization and subsequent washes of the Northern blots with the 18s rRNA DNA primer were carried out 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 106 cells/ml, and allowed to grow to a density of 2–4 × 106 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 Multisizer 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 × 107 cells/ml) and analyzed with a Coulter Multisizer II. A total of 2 × 107 cells were processed for yeast extracts. Cells were washed twice in H2O and then resuspended in 20 μl SDS sample buffer plus protease inhibitors (phenylmethylsulfonyl 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; Harding 1996). Nonsectored colonies, indicative of mutants that may require the plasmid-borne CDC7 gene to survive, were designated lsd for lethal with seven defect. 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 (Kitaeda 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-DC7-AD3-URA3 plasmid), generated two viable and two lethal spores upon tetrad dissection (data not shown). Two 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 PEPE4 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 nonsegregating 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 (=me2-1) 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 (=me2-1) 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 (=me2-1) to all of the currently available cdc7 mutants in our laboratory, and we assayed tetrad s 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 (=me2-1) 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
TABLE 3

Genetic interactions between rad53 and several cell division cycle mutations

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<thead>
<tr>
<th>Strain</th>
<th>Xrad53::11 (PDY258)</th>
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<tr>
<td>A. Interaction between rad53-11 and various cdc7 alleles</td>
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<tr>
<td>Wild type (299)</td>
<td>Viable (10 tetrads)</td>
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<tr>
<td>cdc7-1 (PDY177)</td>
<td>Slow growth phenotype (18 tetrads)</td>
</tr>
<tr>
<td>cdc7-3 (209)</td>
<td>Synthetic lethality/inviable (12 tetrads)</td>
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<tr>
<td>cdc7-4 (576)</td>
<td>Viable (12 tetrads)</td>
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<tr>
<td>cdc7-7 (708)</td>
<td>Synthetic lethality/inviable (33 tetrads)</td>
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<td>B. Interaction between rad53-31 and cdc8-1</td>
<td>Xrad53-31::URA3 (PDY289)</td>
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<td>cdc8-1 (199)</td>
<td>Viable (18 tetrads)</td>
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<td>C. Interaction between rad53-31 and dbf4-1</td>
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<td>dbf4-1 (PDY029)</td>
<td>Slow growth phenotype (31, 24 tetrads)</td>
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</tbody>
</table>

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°C. The rad53-11 and the rad53-31 alleles were marked by a pep4::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 lethal/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.

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. In crosses where the dbf4-1 mutation was the original 31 tetrads dissected, the dbf4-1 mutation was found 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.

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 from the 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 rad53Δ::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 rad53Δ::URA3 disruption was constructed in a heterozygous diploid strain carrying a bob1-1 allele, 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 rad53Δ::URA3 frequently generate second-site suppressors (Zheng et al. 1993; Kim and Weinert 1997; Desany et al. 1998). The number of 3:1 and 4:0 viable lethal tetrads 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 rad53Δ::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 rad53Δ::URA3, indicating that it encodes a functional protein (data not shown). The GAL4-DB-RAD53 (aa50-aa457), which lacks C-terminal sequences, failed to complement the rad53Δ::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; Shollman et al. 1998).

Several attempts were made to show that Dbf4p and Rad53p interact directly using other physical methods. Rad53p/Dbf4p coimmunoprecipitation and GST-Rad53p/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 rad53Δ::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,
revealed a very low level of Dbf4p expression in the rad-53 strain. RAD53 positively regulates Dbf4p. Longer exposures rendered undetectable in the rad53 strain shown in Figure 3. Immunoblots were then probed with antibodies against 7HA-DBF4p and G6PDp. Dbf4p was undetectable in the rad53Δ::hisG strain, indicating that RAD53 positively regulates Dbf4p. Longer exposures revealed a very low level of Dbf4p expression in the rad53Δ::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.

TABLE 4
The bob1-1(mcm5) mutation does not suppress a null mutation in rad53Δ::URA3

<table>
<thead>
<tr>
<th>Relevant genotype of strain dissected</th>
<th>Type of tetrad</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bob1-1 or second site suppressor suppresses rad53Δ::URA3 mutation</td>
<td>4:0 viable:inviable</td>
<td>0</td>
</tr>
<tr>
<td>bob1/ +/+ rad53Δ::URA3 (299 × P119)</td>
<td>3:1 viable:inviable</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2:2 viable:inviable</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Total tetrads dissected</td>
<td>25</td>
</tr>
<tr>
<td>B. Backcross to determine if viable segregants are suppressed by bob1-1 or by a second site suppressor</td>
<td>4:0 viable:inviable</td>
<td>13</td>
</tr>
<tr>
<td>bob1/bob1 +/+rad53Δ::URA3 sup7/+</td>
<td>3:1 viable:inviable</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2:2 viable:inviable</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total tetrads dissected</td>
<td>24</td>
</tr>
</tbody>
</table>

* Viable bob1-1 rad53Δ::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 rad53Δ::hisG strain. However, the POL1 mRNA level was not significantly different between wild-type and rad53Δ::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 rad53Δ::hisG strain, indicating that RAD53 positively regulates Dbf4p. Longer exposures revealed a very low level of Dbf4p expression in the rad53Δ::hisG strain (data not shown). The level ofDbf4p 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 rad53Δ null mutants, the nuclear localization of Rad53p, and the essential function of RAD53 (Zheng et al. 1993). Weinert (1998) suggested that the
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 (=me2-1) 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, Rad53p was shown to interact with Dbf4p in two-hybrid ex-

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.
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.

Experiments. 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
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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