Copyright © 2002 by the Genetics Society of America

Genetic and Physical Interactions Between *DPB11* and *DDC1* in the Yeast DNA Damage Response Pathway

Hong Wang* and Stephen J. Elledge*,†,1

**Verna and Marrs McLean Department of Biochemistry and Molecular Biology and Howard Hughes Medical Institute and* †*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030*

Manuscript received October 2, 2001
Accepted for publication December 10, 2001

ABSTRACT

DPB11 is essential for DNA replication and S/M checkpoint control in *Saccharomyces cerevisiae*. The Dpb11 protein contains four BRCT domains, which have been proposed to be involved in protein-protein interactions. To further investigate the regulation and function of Dpb11, a yeast two-hybrid screen was carried out to identify proteins that physically interact with Dpb11. One positive clone isolated from the screen encoded a carboxyl-terminal fragment of Ddc1 (339–612 aa). Ddc1 is a DNA damage checkpoint protein, which, together with Mec3 and Rad17, has been proposed to form a PCNA-like complex and acts upstream in the DNA damage checkpoint pathways. We further determined that the carboxyl region of Dpb11 is required for its interaction with Ddc1. *DDC1* and *DPB11* also interact genetically. The Δ*ddc1 dpb11-1* double mutant is more UV and MMS sensitive than the Δ*ddc1* or the *dpb11-1* single mutants. Furthermore, the double mutant is more hydroxyurea sensitive and displayed a lower restrictive temperature than *dpb11-1*. These results suggest that *DPB11* and *DDC1* may function in the same or parallel pathways after DNA damage and that *DDC1* may play a role in responding to replication defects.

In the budding yeast *Saccharomyces cerevisiae*, several DNA replication proteins have been shown to be essential for S/M checkpoint control, which inhibits mitotic entry before DNA replication during S-phase is completed (Elledge 1996; Lowndes and Murguia 2000). In *S. cerevisiae*, the S/M checkpoint is assayed by examining the ability of cells to undergo anaphase-like spindle elongation when DNA replication is blocked by hydroxyurea (HU). Pol2, the catalytic subunit of DNA polymerase II (ε), is required for the S/M checkpoint, perhaps by acting as a sensor of DNA replication blocks (Navas *et al.* 1995). Dpb11 physically interacts with Pol2 and is responsible for recruiting Pol2 to DNA replication origins (Masumoto *et al.* 2000). Like Pol2, Dpb11 is also required for the S/M checkpoint (Araki *et al.* 1995; Wang and Elledge 1999). Dpb11 associates with Drc1 (Sld2), another protein required for both DNA replication and S/M checkpoint control (Kamimura *et al.* 1998; Wang and Elledge 1999).

Dpb11 is an evolutionarily conserved protein. Cut5 in *Schizosaccharomyces pombe* (Saka and Yanagida 1993; Saka *et al.* 1994a,b), mus101 in Drosophila (Yamamoto *et al.* 2000), and human TopBP1 (Yamane *et al.* 1997; Yamane and Tsuruo 1999; Makiniemi *et al.* 2001) show sequence and functional similarity to Dpb11. All of them have been shown to be required for DNA replication and with the exception of mus101 are also essential for the S/M checkpoint control. Moreover, TopBP1, like Dpb11, also interacts with DNA polymerase ε (Makiniemi *et al.* 2001).

Dpb11 and its homologs contain *BR*CA1 *c*arboxy-*t*erminal (BRCT) domains, a putative protein-protein interaction motif (Bork *et al.* 1997; Huyton *et al.* 2000). BRCT domains have been identified in >50 proteins involved in DNA repair, recombination, or cell cycle control. X-ray crystallography revealed the three-dimensional structure of the BRCT domain of XRCC1 as a globular motif (Zhang *et al.* 1998). Accumulating evidence suggests that BRCT domains mediate homo/hetero BRCT multimer formation, non-BRCT interactions, and DNA end binding (Huyton *et al.* 2000). For example, XRCC1 forms a heterodimer via its BRCT domain with DNA ligase III (Taylor *et al.* 1998). Rad9, a DNA damage checkpoint protein in *S. cerevisiae*, oligomerizes after DNA damage through its BRCT domain (Soulier and Lowndes 1999). A yeast two-hybrid screen with *S. pombe* Cut5 led to the identification of the Crb2 protein, which is also a BRCT domain-containing checkpoint protein (Saka *et al.* 1997). Recently, it has been shown that TopBP1 interacts with the checkpoint protein hRad9 through its BRCT domains (Makiniemi *et al.* 2001).

To fully understand the function of Dpb11 and to study the mechanism of the S/M checkpoint pathway, we carried out a yeast two-hybrid screen for proteins that physically interact with Dpb11. One of the putative Dpb11 interacting clones encoded the carboxyl terminus of a DNA damage checkpoint protein, Ddc1. We focused our study on Ddc1 because the *S. pombe* homolog of Dpb11, Cut5, was shown to be required for DNA

[1]*Corresponding author:* T307, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030. E-mail: selledge@bcm.tmc.edu

damage checkpoint control (McFarlane *et al.* 1997; Verkade and O'Connell 1998). Therefore we wished to explore the potential role of Dpb11 in response to DNA damage in the context of *DDC1*. Moreover, the function of *DDC1* has been established to some extent. *DDC1* belongs to the *MEC3*, *RAD17*, *RAD24* epistasis group, which, together with *RAD9*, is proposed to act at the beginning of the DNA damage checkpoint pathways (Kondo *et al.* 1999). However, Δ*ddc1* mutants are competent for the S/M checkpoint, although *DDC1* is required for slowing down DNA replication in the presence of DNA damage (Longhese *et al.* 1997), also known as the intra-S-phase checkpoint. Ddc1, Mec3, and Rad17 have been proposed to form a proliferating cell nuclear antigen (PCNA)-like complex. PCNA functions as the sliding clamp that tethers DNA polymerase to its DNA template (Kondo *et al.* 1999; Venclovas and Thelen 2000). On the basis of this structural similarity, it is proposed that the Ddc1-Mec3-Rad17 complex serves as a structural mediator for initiation of checkpoint signaling and provides processivity for DNA repair proteins (Venclovas and Thelen 2000).

We showed here that Ddc1 and Dpb11 not only physically interact, but they also genetically interact with each other. The *dpb11* Δ*ddc1* double mutant is more sensitive to DNA damaging agents and DNA replication inhibitors, suggesting that Dpb11 and Ddc1 might collaborate in responding to DNA abnormalities. Deletion of *DDC1* also lowers the restrictive temperature of the *dpb11* mutant, implying that *DDC1* is required for monitoring any DNA replication defects or DNA damage resulting from *dpb11* mutation.

MATERIALS AND METHODS

DNA plasmids: pHW1 (pAS2-*DPB11*) was constructed by first engineering a *Nde*I site at the start codon of *DPB11* and then subcloning the entire *DPB11* coding sequence from *Nde*I (−3 bp) to *Sal*I (2500 bp) into the *Nde*I/*Sal*I site of the bait vector, pAS2 (Bai and Elledge 1997). *DPB11* coding sequence was cloned into the *Sma*I/*Sac*I site of pBAD98 (Desany *et al.* 1998), generating pHW82 (pBAD98-*DPB11*). The *Hin*dIII/*Sac*I fragment containing the *DPB11* coding region from pHW82 was transferred to the *Hin*dIII/*Sac*I site of pRS415 (Sikorski and Hieter 1989), generating pHW84. The promoter region of *DPB11*, starting from a *Hin*dIII site (−680 bp), was amplified by PCR and a *Nde*I site was engineered just at the start codon of *DPB11*. The resulting PCR product was digested by *Hin*dIII and *Nde*I and cloned into the *Hin*dIII/*Nde*I site of pHW84, generating pHW85 (pRS415-*DPB11* under its own promoter).

Yeast strains: Yeast strains used in this study are isogenic with the W303-derived Y300 strain. All derived strains were constructed using standard genetic crosses and are listed in Table 1. Gene disruptions were performed by replacing one copy of the target gene from a diploid wild-type genome with the *HIS*3 marker (Lorenz *et al.* 1995) and the correct targeting events were confirmed by Southern blotting analysis. Y1187 containing the hemagglutinin (HA)-tagged Ddc1 was obtained by integrating *Pst*I-cleaved pML119 (Longhese *et al.* 1997) into Y300.

Yeast two-hybrid screen: The yeast two-hybrid screen was performed as described (Bai and Elledge 1997). Basically, Y190 was sequentially transformed with pAS2-*DPB11* (pHW1) and a *S. cerevisiae* cDNA-GAL4 activation domain (AD) fusion library. An estimated ~1 million transformants were screened. Yeast clones containing potential Dpb11 interacting proteins were identified by growth on SC-Trp, Leu, His plates with 50 mM 3-amino-1,2,4-triazole (3-AT) (A8056; Sigma, St. Louis) for *HIS*3 transcription. A total of 48 clones were obtained from *HIS*3 selection and 12 of them also turned blue by X-gal colony filter assay for *LacZ* transcription. To eliminate false-positive clones, all the positive clones were transformed back into Y190 with either pAS2 empty vector or other bait plasmids encoding Cdk2, Snf1, lamin, or p53, respectively (Bai and Elledge 1997). All of these combinations did not lead to the activation of either the *HIS3* or *LacZ* reporter gene.

Construction of temperature-sensitive or HU-sensitive *dpb11* mutants: pHW85 (pRS415-*DPB11*) was used as template to carry out the low-fidelity PCR reaction (Wang and Elledge 1999). Primers used in the mutagenesis PCR are as follows: Dpb11-1-1, 5′-CTTCTATTTCTAGTATGGCAGG-3′ (upstream of *DPB11* coding sequence); and Drc1-9, 5′-GTGAGTTACCTCA CTCATTAGGC-3′ (pRS415 vector sequence; Wang and Elledge 1999). The *DPB11*-mN library was generated by replacing the *Nde*I/*Pst*I fragment of pHW85 (including the N-terminal ~770 bp of *DPB11*) by the PCR products and the *DPB11*-mC library was generated by replacing the *Pst*I/*Sac*I fragment of pHW85 (including C-terminal ~1.5 kb of Dpb11 coding sequence) by the PCR products.

The two libraries were screened in YHW186 as described (Wang and Elledge 1999). No temperature-sensitive mutants were isolated from the *DPB11*-mC library. Seven *dpb11* temperature-sensitive (ts) alleles, called pHW164 (*dpb11-9*), pHW165 (*dpb11-13*), pHW166 (*dpb11-3*), pHW167 (*dpb11-2*), pHW168 (*dpb11-11*), pHW169 (*dpb11-4*), and pHW170 (*dpb11-12*), were isolated from the *DPB11*-mN library. Three of these were integrated into yeast strains, generating Y1198 (*dpb11-2*, ts at 34°), Y1199 (*dpb11-3*, ts at 32°), and Y1200 (*dpb11-4*, ts at 30°). Four new HU-sensitive *dpb11* alleles were isolated, two from the *DPB11*-mN library and two from the *DPB11*-mC library. They were named pHW160 (*dpb11-5*), pHW161 (*dpb11-6*), pHW 162 (*dpb11-7*), and pHW163 (*dpb11-8*).

UV sensitivity measurement: Approximately 500 log-phase cells were spread on plates and then treated with different doses of UV light. UV sensitivity was measured by counting the colonies formed after several days.

RESULTS

Dpb11 interacts with the C terminus of Ddc1: Dpb11 contains four BRCT domains that are likely to mediate protein-protein interactions. To explore possible Dpb11-binding proteins, we carried out a yeast two-hybrid screen for Dpb11. The bait plasmid, pAS2-*DPB11*, was constructed by fusing the entire Dpb11 protein to the C terminus of the GAL4 DNA-binding (DB) domain. We first confirmed that the fusion protein encoded by pAS2-*DPB11* is properly expressed and functions as wild-type Dpb11 because it could complement the growth of *dpb11* null cells (Figure 1A).

A *S. cerevisiae* cDNA GAL4AD fusion library was screened. Twelve clones were isolated from an estimated ~1 million transformants as positive for the reporter gene activity (His+ LacZ+). Two out of the 12 positive

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
Y300	*MAT***a** *trp1-1 ura3-1 his3-11,15 leu2-3,112 ade2-1 can1-100*	Allen *et al.* (1994)
Y1185	As Y300, *dpb11-1*	This study
Y1135	As Y300, Δ*ddc1*	Alcasabas *et al.* (2001)
Y438	As Y300, Δ*rad9*	Navas *et al.* (1996)
Y1186	As Y300, Δ*dpb11*:His + pHW82 (pBAD98-*DPB11*)	This study
Y1187	As Y300, *DDC1-HA2*:Leu	This study
Y1188	As Y300, Δ*dpb11*:His + pHW85 (pRS415-*DPB11*)	This study
Y1189	As Y300, *dpb11-1* Δ*ddc1*	This study
Y1190	As Y300, *dpb11-1* Δ*rad9*	This study
Y1191	As Y300, Δ*rad17*	This study
Y1192	As Y300, Δ*mec3*	This study
Y1193	As Y300, Δ*rad24*	This study
Y1194	As Y300, *dpb11-1* Δ*rad17*	This study
Y1195	As Y300, *dpb11-1* Δ*rad24*	This study
Y1196	As Y300, *dpb11-1* Δ*mec3*	This study
Y1197	As Y300, *dpb11-1* Δ*xrs2*	This study
Y1198	As Y300, *dpb11-2*	This study
Y1199	As Y300, *dpb11-3*	This study
Y1200	As Y300, *dpb11-4*	This study
Y1201	As Y300, *dpb11-2* Δ*ddc1*	This study
Y1202	As Y300, *dpb11-3* Δ*ddc1*	This study
Y1203	As Y300, *dpb11-4* Δ*ddc1*	This study
Y799	As Y300, *drc1-1*	Wang *et al.* (1999)

clones encoded the C-terminal 274 residues of Ddc1, named Ddc1-C (Figure 1B).

The C-terminal region of Dpb11 is responsible for its interaction with Ddc1: To identify the region of Dpb11 that is responsible for its interaction with Ddc1, two truncated forms of Dpb11 were fused to the GAL4-DB domain. Each contains two BRCT domains and they are named Dpb11-N [1–256 amino acids (aa)] and Dpb11-C (251–764 aa), respectively (Figure 1C). By examining the activation of the reporter genes, we found that Dpb11-C, but not Dpb11-N, interacted with Ddc1. Although no interaction between full-length Dpb11 and Ddc1 could be detected, Dpb11-C interacts with both full-length Ddc1 and Ddc1-C. Interestingly, *dpb11-1*, which encodes a C-terminal truncated protein (Kamimura *et al.* 1998), is also defective in interacting with Ddc1. This result further supported the interpretation that the C terminus of Dpb11 is responsible for its interaction with Ddc1.

Dpb11 and Ddc1 physically interact with each other *in vitro*: To test if we could detect a physical association between Dpb11 and Ddc1, we tried both *in vivo* and *in vitro* methods. We were unable to co-immunoprecipitate these proteins *in vivo* in untreated, methyl methane sulfonate (MMS)-, or HU-treated cells. We reasoned that their association might be too weak to survive immunoprecipitation conditions or it may occur on chromatin. Thus we tested whether Dpb11 and Ddc1 interact with each other *in vitro*. An *in vitro* glutathione *S*-transferase (GST) pull-down experiment was performed. GST-Dpb11, but not GST, could bind HA-tagged Ddc1 from the yeast extract (Figure 2A), indicating that Dpb11 interacts with Ddc1 *in vitro*.

If Ddc1 physically interacts with Dpb11, we would expect genetic interactions between them as well. Therefore, we examined interactions between mutations of *DDC1* and *DPB11* and other components of the *DPB11* pathway. It has been shown that Dpb11 physically interacts with Drc1, another DNA replication and S/M checkpoint protein (Kamimura *et al.* 1998; Wang and Elledge 1999). We observed that overexpression of Ddc1 is toxic to *drc1-1* mutants and this toxicity can be reversed when *DPB11* is co-overexpressed (Figure 2B). The toxicity of overexpression of *DDC1* is specific to *drc1-1* cells. *DDC1* overexpression does not confer toxicity in wild-type or *dpb11-1* cells (data not shown). Thus, the toxicity is not general. A plausible explanation for these observations is that Dpb11 interacts with both Ddc1 and Drc1 and overexpressed Ddc1 competes with Drc1 for Dpb11, therefore resulting in toxicity in *drc1-1*. Co-overexpression of *DPB11* alleviates this competition and relieves the toxicity.

Genetic interactions between *DPB11* and *DDC1*: Ddc1 is essential for DNA damage checkpoint control and a Δ*ddc1* mutant is very sensitive to UV irradiation (Longhese *et al.* 1997). *dpb11-1* mutants have also been shown to be slightly sensitive to UV (Araki *et al.* 1995). We found that the Δ*ddc1 dpb11* double mutant is more UV and MMS sensitive than either single mutant (Figure 3A). To test if *DPB11* and *DDC1* share some redundant

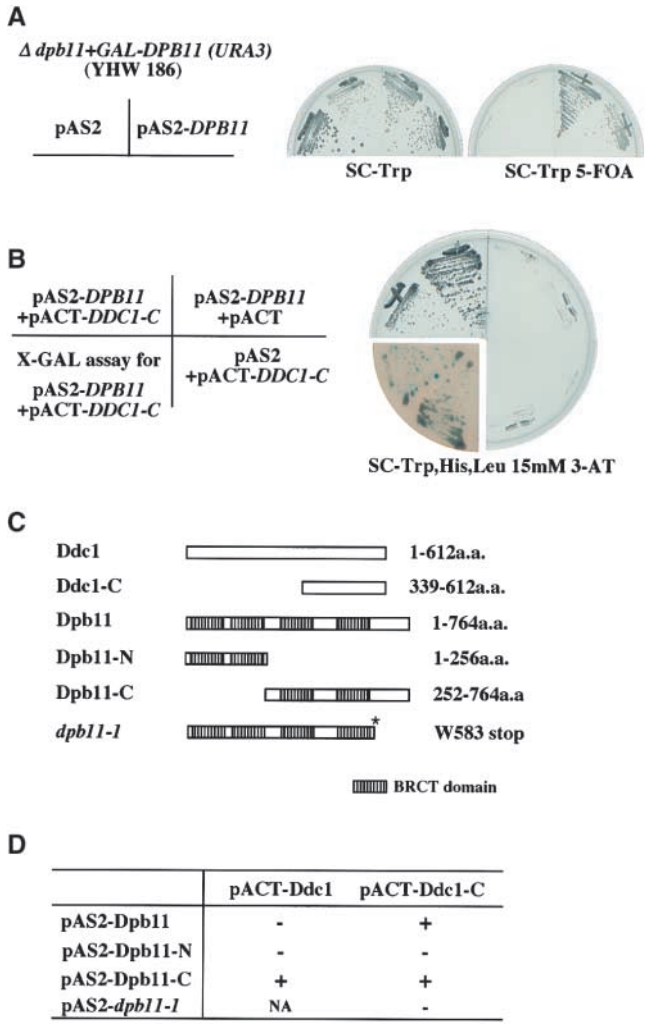

FIGURE 1.—Dpb11 and Ddc1 interact with each other through their C termini. (A) pAS2-*DPB11*, which encodes the GAL4 DNA-binding domain-Dpb11 fusion protein, can suppress the lethality of Δ*dpb11*. Y1186, a *dpb11* null strain containing a pHW82 (pBAD98-*DPB11*, *URA3*) plasmid, was transformed with pAS2 or pAS2-*DPB11* and then streaked on SC-Trp or SC-Trp plates containing 5-fluoroorotic acid (5-FOA). pAS2-*DPB11* could support this strain to grow on a 5-FOA plate. (B) The C terminus of Ddc1 (339–612 aa) interacts with Dpb11 in the yeast two-hybrid system. Strain Y190 carrying the plasmids encoding the bait and/or prey proteins as indicated was incubated on SC-Trp, Leu, His plates containing 15 mM 3-AT to test for activation of the *HIS3* reporter and the colonies were also tested for LacZ transcription by X-gal assay. (C and D) The C-terminal region of Dpb11 is required for interaction with Ddc1. Strain Y190 was transformed with the constructs indicated in C. Protein-protein interaction was assessed by the growth of transformants on 15 mM 3-AT and by X-gal assays. The results are listed in D.

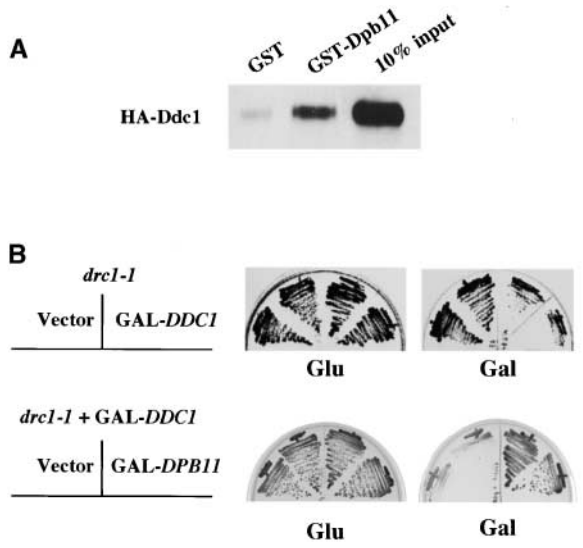

FIGURE 2.—(A) Ddc1 interacts with Dpb11 *in vitro*. GST or GST-Dpb11 was purified from baculovirus-infected insect cells using glutathione beads. Approximately 0.5 μg of purified proteins bound on glutathione beads was incubated with 400 μg of yeast protein extract from Y1187 (*DDC1-HA*) at 4° for 2 hr. Beads were washed and protein samples were analyzed by Western blotting. Ddc1 was detected using anti-HA antibodies. (B) Overexpression of *DDC1* in *drc1-1* is toxic and this toxicity can be reversed by co-overexpression of *DPB11*. *drc1-1* (Y799) cells were transformed with vector alone or pGAL-*DDC1* (pML109; LONGHESE *et al.* 1997). The transformants were grown on either glucose or galactose plates as indicated and incubated at 24° (top). *drc1-1* carrying pGAL-*DDC1* were transformed with pGAL-*DPB11* and the growth of the transformants was tested on either glucose or galactose plates as indicated (bottom).

functions and might mutually suppress the defects of each other, we overexpressed *DPB11* in Δ*ddc1* mutants and vice versa. No suppression of the UV sensitivity of Δ*ddc1* was observed with overexpressed *DPB11* (data not shown). When *DDC1* was overexpressed in *dpb11-1* cells, a partial suppression of the UV sensitivity of *dpb11-1* mutants was observed (Figure 3B), suggesting that *DPB11* and *DDC1* function in the same or parallel pathways in response to DNA damage.

Dpb11 is not essential for the DNA-damage-induced hyperphosphorylation of Ddc1: Ddc1 is hyperphosphorylated after DNA damage in a *MEC1*-dependent manner (LONGHESE *et al.* 1997; PACIOTTI *et al.* 1998). To test if Dpb11 is required for the phosphorylation of Ddc1 after DNA damage, wild-type and *dpb11-1* cells were treated with UV (50 J/m^2), and then the extent of Ddc1 phosphorylation was examined by Western blot. However, Ddc1 phosphorylation in *dpb11-1* mutants was not reduced compared to that in wild-type cells, suggesting that Dpb11 is not essential for DNA damage-induced Ddc1 phosphorylation and is not functioning upstream in that capacity (data not shown).

***dpb11-1* mutants are proficient for the G2/M DNA damage checkpoint:** The genetic interaction between *DPB11* and *DDC1* suggests that Dpb11 might play a role in response to DNA damage. Pol2 has been shown to be important for the DNA damage checkpoint control in S-phase cells, while Rad9 mainly functions in cells outside of S-phase (NAVAS *et al.* 1996). It is possible that Dpb11 is also essential for DNA damage checkpoint control in S-phase only. However, when α-factor-arrested

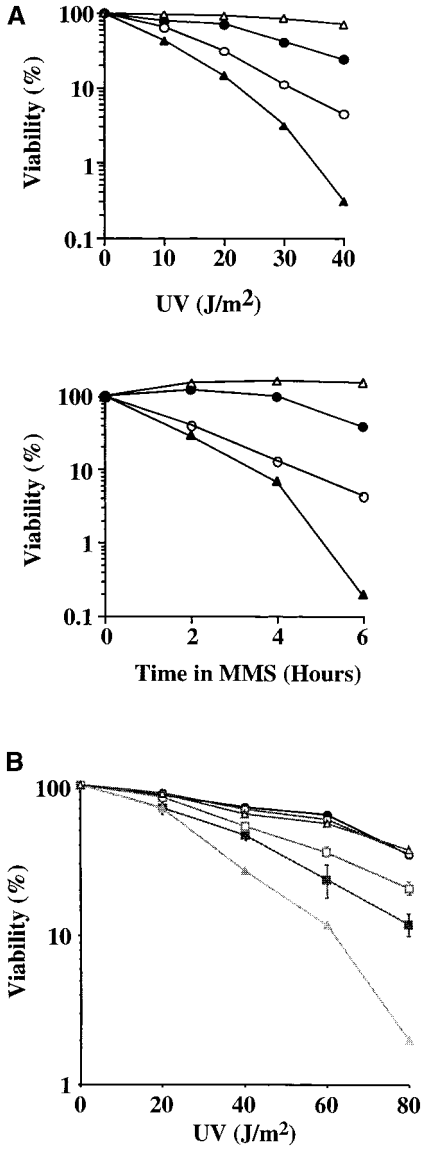

FIGURE 3.—Genetic interactions between *DPB11* and *DDC1*. (A) Δ*ddc1 dpb11-1* double mutants showed enhanced UV-sensitive and MMS-sensitive phenotype. Log-phase (△) wild-type (Y300), (○) Δ*ddc1* (Y1135), (●) *dpb11-1* (Y1185), and (▲) Δ*ddc1 dpb11-1* (Y1189) cells were spread on YPD plates and UV irradiated at different doses. Viability was scored by counting the colonies that grew up on the plates after 3 days at 24° (top). Log-phase wild-type (Y300), Δ*ddc1* (Y1135), *dpb11-1* (Y1185), and Δ*ddc1 dpb11-1* (Y1189) cells were treated with 0.01% MMS. Aliquots were withdrawn at the indicated times to test viability (bottom). (B) Overexpression of *DDC1* slightly suppresses the UV sensitivity of *dpb11-1* cells. (●, ○) Wild-type (Y300), (■, □) *dpb11-1* (Y1185), and (▲, △) Δ*ddc1* (Y1135) cells, harboring either empty vector (solid) or GAL-*DDC1* (open), were cultured to log phase in galactose medium. Cells were spread on SC-Ura, Gal plates and treated with different doses of UV light and cell viability was determined as in A.

or nocodazole-arrested *dpb11-1* cells were irradiated by UV light, they were still more sensitive to UV than to wild-type cells (Figure 4A), suggesting that Dpb11 also functions outside of S-phase.

S. pombe Cut5 has been implicated in DNA damage checkpoint control. A role in controlling cell cycle transitions after DNA damage could explain the UV sensitivity of *dpb11-1* in G2. To test this, wild-type, Δ*ddc1*, and *dpb11-1* cells were arrested in G2 by nocodazole treatment and shifted to 36° for 30 min to inactivate the *dpb11-1* mutant. Then, the cells were irradiated with UV and released at 36°. The percentage of cells that had one nucleus was counted to monitor the anaphase entry. As reported previously (LONGHESE *et al.* 1997), Δ*ddc1* is DNA damage checkpoint defective, as ~40% of cells entered anaphase in the presence of DNA damage. In contrast, *dpb11-1* mutant cells behaved like wild-type cells and maintained cell cycle arrest. Therefore, they are proficient for cell cycle arrest after DNA damage, and their UV-sensitivity phenotype during G2 is more likely to result from a DNA repair defect.

***DDC1* plays a role in response to DNA replication defects:** In experiments designed to examine genetic interactions between *DPB11* and *DDC1* in response to S-phase stress, we observed that double mutants between the *DDC1* group of genes (*DDC1*, *MEC3*, and *RAD17*) and *dpb11-1* are much more sensitive to HU than either of the single mutants. In contrast to the *DDC1* group of genes, Δ*rad9 dpb11-1* double mutants did not have a dramatic additive HU-sensitive phenotype (Figure 5). This observation suggested that *DDC1* might play a role in response to DNA replication defects.

In addition, mutations in *DDC1* lowered the restrictive temperature of *dpb11-1* (Figure 6), indicating that DNA damage checkpoint or some aspect of Ddc1 function is required for the survival of *dpb11-1* at higher temperatures. Since *DDC1* has not been shown to be involved in either DNA replication or the S/M checkpoint, it is possible that the defects of *dpb11-1* introduce DNA damage during S-phase, which requires the DNA damage checkpoint response pathway. If this is the case, then proteins involved in DNA damage repair will also be required for *dpb11-1*'s survival. Double mutants between *dpb11* and Δ*rad51* or Δ*xrs2*, DNA damage repair mutants, were constructed and observed for an exacerbated phenotype. The *dpb11* Δ*xrs2* double mutants did have a lower restrictive temperature than *dpb11-1*, but the *dpb11* Δ*rad51* mutants did not (Figure 6 and data not shown). However, in addition to its DNA damage repair function, *XRS2* has also been shown to be important in some aspect of checkpoint control (D'AMOURS and JACKSON 2001; GRENON *et al.* 2001; USUI *et al.* 2001). Therefore, the genetic interactions we observed between *DPB11* and *XRS2* might also be due to the defective checkpoint control in *xrs2* cells.

Novel alleles of *DBP11* reveal a linkage between DNA replication defects and S/M checkpoint defects: We wished to determine whether the genetic interactions we observed between *dpb11-1* and Δ*ddc1* mutants were allele specific or reflected a general need for *DDC1* function in response to an absence of *DPB11* function. However, there was only one allele of *DBP11*, *dpb11-1*, which is both ts and HU sensitive. Since *DPB11* functions in both DNA replication and the S/M checkpoint path-

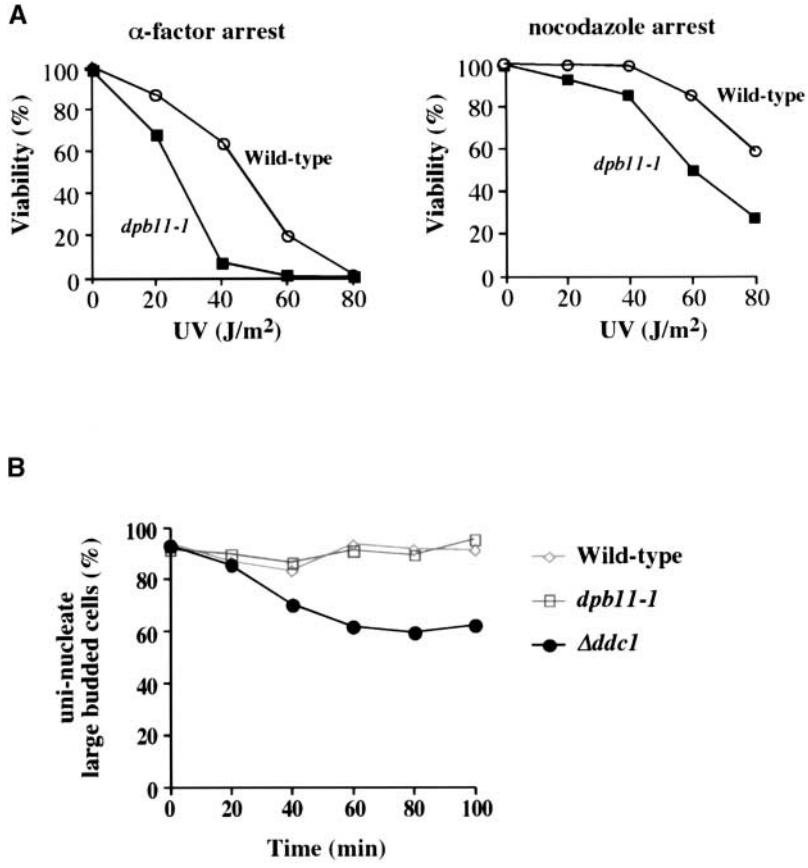

FIGURE 4.—*dpb11-1* is proficient for the G2/M DNA damage checkpoint. (A) G1- and G2-arrested *dpb11-1* cells are UV sensitive. α-factor or nocodazole-arrested wild-type (Y300) and *dpb11-1* (Y1185) cells were cultured on YPD plates and UV irradiated at indicated doses. (B) Log-phase wild-type (Y300), *dpb11-1* (Y1185), and Δ*ddc1* (Y1135) cells were arrested with 10 μg/ml nocodazole and irradiated with 50 J/m^2 UV. At the indicated times, the percentages of uninucleate large-budded cells were scored by 4′,6-diamidino-2-phenylindole staining.

ways in *S. cerevisiae* and the *dpb11-1* allele is defective for both of these functions, it was unclear which of these defects needed *DDC1* function. We thus carried out a screen for additional *dpb11* alleles, trying to separate the two functions of Dpb11 by mutation.

We independently mutagenized the N terminus and the C terminus of Dpb11 and the resulting mutagenized libraries, *DPB11*-mN and *DPB11*-mC, were screened for either ts or HU-sensitive *dpb11* mutants (see MATERIALS AND METHODS). Four HU-sensitive alleles were isolated with similar HU sensitivity as *dpb11-1*; however, all were also ts (data not shown). Seven new ts alleles of *dpb11* were isolated when the *DPB11*-mN library was used and all were HU sensitive. Three of them were integrated into the genome. Interestingly, all three new ts alleles, *dpb11-2*, *dpb11-3*, and *dpb11-4*, elongated their spindles like *dpb11-1* after 2 hr when cultured at 37°. Furthermore, all three mutants lost ~90% viability after 4 hr at 37° (data not shown). These results suggested that they are also defective in the S/M checkpoint.

We mapped the mutation sites for all the *dpb11-1* alleles we isolated (Table 2). However, no common residues were mutated in these mutants. Several of the mutated amino acids are conserved residues in the BRCT domains and some are conserved between Dpb11 and Cut5. It is possible that the mutations at those sites structurally interfere with the function of Dpb11.

We were unable to obtain specific S/M checkpoint-defective or DNA replication-defective *dpb11-1* mutant alleles, indicating that unlike Pol2, the DNA replication function and S/M checkpoint function of Dpb11 are unlikely to be separated by mutation.

The genetic interactions between *DPB11* and *DDC1* are not allele specific: With three new ts alleles available, we then examined whether the genetic interactions between *DPB11* and *DDC1* are allele specific by crossing *dpb11-2*, *dpb11-3*, and *dpb11-4* mutants with Δ*ddc1* mutants. The resulting double mutants were each more temperature sensitive than *dpb11* single mutants, indicating that the interaction between these two genes is not allele specific and reflects a general defect common to each *dpb11* allele (Figure 6).

DISCUSSION

Dpb11 is an essential gene that is required for both DNA replication and S/M checkpoint controls (ARAKI *et al.* 1995; WANG and ELLEDGE 1999). The fact that we failed to separate these two functions of Dpb11 by mutation strongly suggests that the two functions are connected with each other. Our studies argue against the model that Dpb11 has two domains that have separate and independent functions; instead, it is likely that the S/M checkpoint function of Dpb11 is directly linked to its DNA replication function. In addition, as a number of other proteins [Drc1 (Sld2), Orc1, and Rfc1] involved

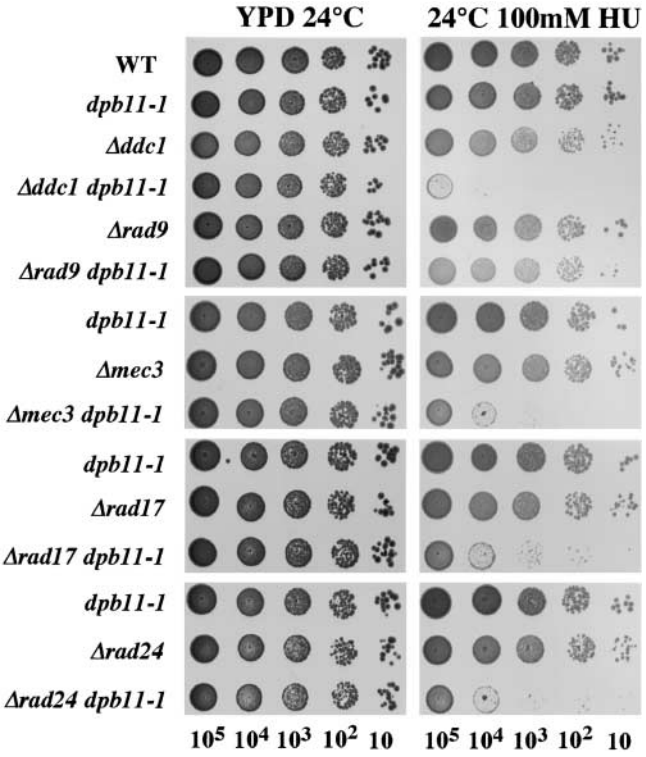

FIGURE 5.—Deletion of *DDC1*, *MEC3*, and *RAD17*, but not *RAD9*, makes *dpb11-1* more HU sensitive. Cells of the indicated genotypes were cultured to log phase, and then 10-fold serial dilutions of cells were spotted onto either YPD plates or YPD with 0.1 M HU and incubated at 24° for several days.

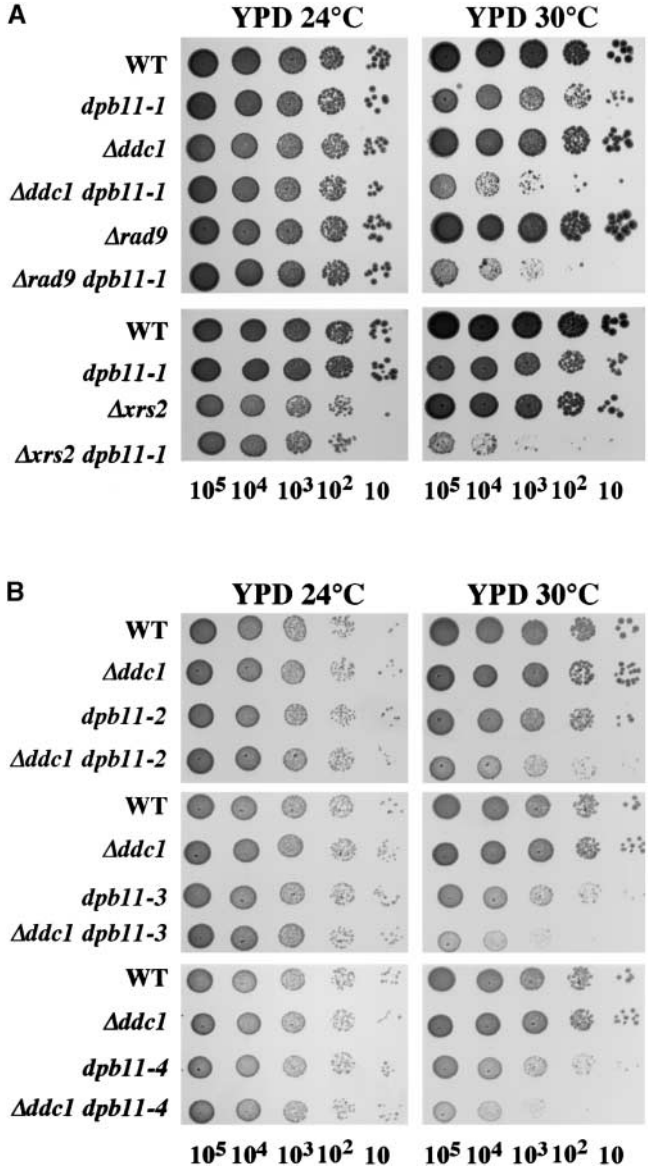

FIGURE 6.—DNA damage checkpoint genes and DNA damage repair genes are required for the survival of *dpb11-1* at higher temperature. (A) Cells of the indicated genotypes were cultured to log phase, and then 10-fold serial dilutions of cells were spotted onto YPD plates and incubated at the indicated temperatures. (B) Double mutants between Δ*ddc1* and different *dpb11* alleles are more temperature sensitive than *dpb11* single mutants.

in initiation of DNA replication have S/M checkpoint defects when treated with HU, it is likely that the deficiency responsible for this is their defect in DNA replication. One could envision a model in which fewer active replication forks give rise to a proportionally lower checkpoint signal. Once the level of DNA replication drops below the threshold needed to activate enough Rad53 in response to HU to arrest the cell cycle, a checkpoint defect occurs. In this threshold model, these initiator proteins would have only indirect roles in transducing the replication stress signals.

Dpb11 contains four BRCT domains that are believed to be important for mediating protein-protein interaction. In an attempt to identify proteins that physically interact with Dpb11, we carried out a yeast two-hybrid screen. One of the positive clones encodes the C terminus of the DNA damage checkpoint protein Ddc1 (LONGHESE *et al.* 1997). Ddc1 interacts with the C terminus of Dpb11 containing the third and fourth BRCT repeats. Interestingly, the *dpb11-1* mutation, which encodes a C-terminal truncated form of the Dpb11 protein, is defective for the interaction with Ddc1, further supporting the notion that the C terminus of Dpb11 is important for its interaction with Ddc1. We failed to detect an interaction between Dpb11 and Ddc1 by coimmunoprecipitation experiments. Since Dpb11 interacts with Ddc1 *in vitro*, we speculate that the *in vivo* interaction between Dpb11 and Ddc1 is normally regulated and their interaction may occur only transiently under special circumstances, such as on chromatin at a stalled replication fork.

What is the significance of the interaction between Dpb11 and Ddc1? One possibility is that *DPB11* plays a role in response to DNA damage, where it utilizes Ddc1 in some capacity. *dpb11-1* mutants are sensitive to various DNA damaging agents, such as UV and MMS, even outside of S-phase. However, we did not observe cell

TABLE 2

Mutation site-mapping results of the ts and HU-sensitive *dpb11* alleles

dpb11 alleles	No. of amino acids	From	To	Conserved in BRCT domain	Conserved in Cut5
dpb11-5	209	Arg	Gly	N[a]	Y[a]
dpb11-6	55	Lys	Glu	N	Y
	166	Asp	Gly	N	N
	243	Ile	Val	N	N
dpb11-7	280	Phe	Ser	N	N
	325	Leu	Ser	N	N
	333	Ile	Asn	Y	N
	459	Leu	Ser	N	N
	471	Met	Val	N	N
	526	Asn	Asp	N	N
	551	Phe	Leu	N	N
	573	Asn	Ile	N	N
	598	Lys	Arg	N	N
	636	His	Asp	N	N
	712	Gln	Arg	N	N
	729	Ser	Pro	N	N
	738	Ile	Val	N	Y
	759	Thr	Ala	N	N
	763	Asp	Gly	N	N
dpb11-8	280	Phe	Ser	N	N
	405	Leu	Ser	Y	Y
dpb11-9	22	Lys	Arg	N	N
	48	Gly	Trp	N	Y
	83	Ser	Pro	N	N
dpb11-13	32	Gly	Ser	Y	N
dpb11-3	213	Leu	Ser	Y	Y
dpb11-2	65	Asp	His	N	Y
	74	Ile	Val	Y	Y
	226	Asp	Tyr	N	Y
dpb11-11	53	Thr	Pro	N	Y
	71	Ile	Thr	N	N
	130	Asn	Asp	N	N
	182	Leu	Ser	N	N
	197	Ile	Val	Y	N
dpb11-4	220	Leu	Pro	Y	N
	242	Lys	Glu	N	N
dpb11-12	56	Phe	Ser	N	N
	145	Gly	Glu	Y	N

[a] Y, conserved; N, not conserved.

cycle arrest defects in *dpb11-1* mutants after DNA damage and there is no significant additive phenotype in terms of activation of Rad53 phosphorylation in *dpb11-1* Δ*ddc1* double mutants after DNA damage (data not shown). These results indicate that Dpb11 is not essential for DNA damage checkpoint signaling. The *S. pombe* homolog of Cut5 has been shown to be required for DNA damage checkpoint signaling (McFarlane *et al.* 1997; Verkade and O'Connell 1998). If *DPB11* plays a role in DNA damage checkpoint signaling, it is likely to be minor and is unlikely to explain *DBP11*'s DNA damage sensitivity.

Our data and other published reports suggest that Dpb11 might be involved in DNA repair. This is supported by several lines of evidence. First, Dpb11 physically interacts with Pol2, the catalytic subunit of DNA polymerase ε (Masumoto *et al.* 2000). Pol2 has been shown to be involved in nucleotide excision repair, DNA double-strand break repair, and other types of repair (Wang *et al.* 1993; Aboussekhra *et al.* 1995; Budd and Campbell 1995; Kramata *et al.* 1998; Holmes and Haber 1999). Biochemical studies also indicate that Dpb11 is responsible for recruiting Pol2 to origins during DNA replication (Masumoto *et al.* 2000). Thus, it

is possible that Dpb11 collaborates with Pol2 during the DNA damage repair process or potentially recruits Pol2 to sites of damage. Second, our studies indicated strong genetic interactions between *DPB11* and DNA damage checkpoint genes. Since the *dpb11* mutant appears proficient for arresting the cell cycle after DNA damage, *dpb11-1*'s DNA damage sensitivity suggests it is likely to be involved in the damage repair process. Finally, it has been shown that the human homolog of Dpb11, TopBP1, binds DNA breaks *in vitro* (Yamane and Tsuruo 1999) and co-localizes with Brca1 after DNA damage *in vivo* (Makiniemi *et al.* 2001), suggesting a role for TopBP1, and indirectly for Dpb11, in DNA repair. Interestingly, TopBP1 also physically interacts with hRad9, the human homolog of Ddc1 (Makiniemi *et al.* 2001). It has been proposed that after DNA damage, the Ddc1/Mec3/Rad17 complex, in addition to sensing the damage signal, might also recruit DNA damage repair proteins to the sites of DNA damage. Therefore, given the finding that Ddc1 interacts with Dpb11, it is possible that Dpb11 is one of the repair proteins that is recruited by Ddc1.

Another explanation for the Ddc1-Dpb11 interaction is that Ddc1 might collaborate with Dpb11 in response to DNA replication defects. Although *DDC1* is not essential for DNA replication, it was identified from a synthetic lethal screen with a primase subunit mutant, suggesting Ddc1 might have a role in monitoring DNA replication. Consistent with this model, Δ*ddc1 dpb11-1* double mutants are much more HU sensitive than either single mutant. This is true for other mutants in the *DDC1* epistasis group such as *rad17*, *rad24*, and *mec3*. The need for *DDC1* could be explained if HU treatment of *dpb11* mutants induced DNA damage and therefore required *DDC1*'s role in checkpoint control. However, *rad9* mutants, which are equally defective in DNA damage checkpoint control as *ddc1* mutants, do not enhance the HU sensitivity of *dpb11* mutants, which suggests that it is not the checkpoint function of *DDC1* that is required for survival of *dpb11* mutants experiencing replication stress. Instead these results argue that Ddc1 also plays a role after DNA replication is compromised, and this role is not related to cell cycle arrest. Therefore, we suspect that *DDC1* is playing a role in some aspect of repair at disrupted replication forks. The Ddc1-Mec3-Rad17 complex has previously been implicated in DNA repair because rates of excision of DNA from telomere regions in *cdc13* mutants are significantly reduced in *mec3* and *rad17* mutants (Lydall and Weinert 1995), suggesting they might recruit nucleases to telomeric regions of DNA in the absence of Cdc13 function. Recruitment of nucleases or other repair proteins such as helicases could facilitate the repair of damaged replication forks.

In contrast to the case of DNA replicational stress, both *DDC1* and *RAD9* are required for the survival of *dpb11* mutants at higher temperatures. This could be explained if *dpb11* mutants activate the DNA damage checkpoint. However, shifting *dpb11-1* mutant cells to the nonpermissive temperature does not result in a mobility shift of Rad53, Rad9, and Ddc1 proteins (data not shown) that normally occurs when the DNA damage checkpoint is activated. This suggests that very little DNA damage is generated in *dpb11* mutants at the nonpermissive temperature. However, we cannot rule out the possibility that at intermediate temperatures in *dpb11* mutants, some DNA damage is made and requires *DDC1*- and *RAD9*-dependent checkpoint signaling for survival. In contrast, in the case of HU-induced DNA replicational stress, Ddc1 plays a role distinct from that of Rad9. This role is more likely to be one of assisting repair rather than controlling cell cycle arrest.

The models we have proposed are not mutually exclusive; it is possible that they all partially reflect some *in vivo* situations depending on cell cycle stages and specific environments. Our data have demonstrated important physical and genetic interactions between Dpb11 and Ddc1. However, more detailed studies will be required to further understand the biochemical significance of these interactions at the molecular level.

We thank H. Ariki, M.P. Longhese, and G. Lucchini for strains and plasmids. We thank members of the Elledge lab for comments, helpful discussion, and/or reagents. This work was supported by National Institutes of Health grant GM44664 (to S.J.E.). S.J.E is an Investigator with the Howard Hughes Medical Institute and a Welch Professor of Biochemistry.

LITERATURE CITED

Aboussekhra, A., M. Biggerstaff, M. K. Shivji, J. A. Vilpo, V. Moncollin *et al.*, 1995 Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell **80:** 859–868.

Alcasabas, A. A., A. J. Osborn, J. Bachant, F. Hu, P. J. H. Werler *et al.*, 2001 Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat. Cell Biol. **3:** 958–965.

Allen, J. B., Z. Zhou, W. Siede, E. C. Friedberg and S. J. Elledge, 1994 The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8:** 2401–2415.

Araki, H., S. H. Leem, A. Phongdara and A. Sugino, 1995 Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc. Natl. Acad. Sci. USA **92:** 11791–11795.

Bai, C., and S. J. Elledge, 1997 Gene identification using the yeast two-hybrid system. Methods Enzymol. **283:** 141–156.

Bork, P., K. Hofmann, P. Bucher, A. F. Neuwald, S. F. Altschul *et al.*, 1997 A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. **11:** 68–76.

Budd, M. E., and J. L. Campbell, 1995 DNA polymerases required for repair of UV-induced damage in Saccharomyces cerevisiae. Mol. Cell. Biol. **15:** 2173–2179.

D'Amours, D., and S. P. Jackson, 2001 The yeast Xrs2 complex functions in S phase checkpoint regulation. Genes Dev. **15:** 2238–2249.

Desany, B. A., A. A. Alcasabas, J. B. Bachant and S. J. Elledge, 1998 Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev. **12:** 2956–2970.

Elledge, S. J., 1996 Cell cycle checkpoints: preventing an identity crisis. Science **274:** 1664–1672.

Grenon, M., C. Gilbert and N. F. Lowndes, 2001 Checkpoint activation in response to double-strand breaks requires the Mre11/Rad50/Xrs2 complex. Nat. Cell Biol. **3:** 844–847.

Holmes, A. M., and J. E. Haber, 1999 Double-strand break repair in

yeast requires both leading and lagging strand DNA polymerases. Cell **96:** 415–424.

Huyton, T., P. A. Bates, X. Zhang, M. J. Sternberg and P. S. Freemont, 2000 The BRCA1 C-terminal domain: structure and function. Mutat. Res. **460:** 319–332.

Kamimura, Y., H. Masumoto, A. Sugino and H. Araki, 1998 Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication. Mol. Cell. Biol. **18:** 6102–6109.

Kondo, T., K. Matsumoto and K. Sugimoto, 1999 Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway. Mol. Cell. Biol. **19:** 1136–1143.

Kramata, P., K. M. Downey and L. R. Paborsky, 1998 Incorporation and excision of 9-(2-phosphonylmethoxyethyl)guanine (PMEG) by DNA polymerase delta and epsilon in vitro. J. Biol. Chem. **273:** 21966–21971.

Longhese, M. P., V. Paciotti, R. Fraschini, R. Zaccarini, P. Plevani *et al.*, 1997 The novel DNA damage checkpoint protein ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast. EMBO J. **16:** 5216–5226.

Lorenz, M. C., R. S. Muir, E. Lim, J. McElver, S. C. Weber *et al.*, 1995 Gene disruption with PCR products in Saccharomyces cerevisiae. Gene **158:** 113–117.

Lowndes, N. F., and J. R. Murguia, 2000 Sensing and responding to DNA damage. Curr. Opin. Genet. Dev. **10:** 17–25.

Lydall, D., and T. Weinert, 1995 Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science **270:** 1488–1491.

Makiniemi, M., T. Hillukkala, J. Tuusa, K. Reini, M. Vaara *et al.*, 2001 BRCT domain-containing protein TopBP1 functions in DNA replication and damage response. J. Biol. Chem. **6:** 6.

Masumoto, H., A. Sugino and H. Araki, 2000 Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol. Cell. Biol. **20:** 2809–2817.

McFarlane, R. J., A. M. Carr and C. Price, 1997 Characterisation of the Schizosaccharomyces pombe rad4/cut5 mutant phenotypes: dissection of DNA replication and G2 checkpoint control function. Mol. Gen. Genet. **255:** 332–340.

Navas, T. A., Z. Zhou and S. J. Elledge, 1995 DNA polymerase epsilon links the DNA replication machinery to the S phase checkpoint. Cell **80:** 29–39.

Navas, T. A., Y. Sanchez and S. J. Elledge, 1996 RAD9 and DNA polymerase epsilon form parallel sensory branches for transducing the DNA damage checkpoint signal in Saccharomyces cerevisiae. Genes Dev. **10:** 2632–2643.

Paciotti, V., G. Lucchini, P. Plevani and M. P. Longhese, 1998 Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein ddc1p, which physically interacts with mec3p. EMBO J. **17:** 4199–4209.

Saka, Y., and M. Yanagida, 1993 Fission yeast cut5+, required for S phase onset and M phase restraint, is identical to the radiation-damage repair gene rad4+. Cell **74:** 383–393.

Saka, Y., P. Fantes, T. Sutani, C. McInerny, J. Creanor *et al.*, 1994a Fission yeast cut5 links nuclear chromatin and M phase regulator in the replication checkpoint control. EMBO J. **13:** 5319–5329.

Saka, Y., P. Fantes and M. Yanagida, 1994b Coupling of DNA replication and mitosis by fission yeast rad4/cut5. J. Cell Sci. **18** (Suppl.): 57–61.

Saka, Y., F. Esashi, T. Matsusaka, S. Mochida and M. Yanagida, 1997 Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and Chk1. Genes Dev. **11:** 3387–3400.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122:** 19–27.

Soulier, J., and N. F. Lowndes, 1999 The BRCT domain of the *S. cerevisiae* checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. Curr. Biol. **9:** 551–554.

Taylor, R. M., B. Wickstead, S. Cronin and K. W. Caldecott, 1998 Role of a BRCT domain in the interaction of DNA ligase III-alpha with the DNA repair protein XRCC1. Curr. Biol. **8:** 877–880.

Usui, T., H. Ogawa and J. H. Petrini, 2001 A DNA damage response pathway controlled by Tel1 and the Mre11 complex. Mol. Cell **7:** 1255–1266.

Venclovas, C., and M. P. Thelen, 2000 Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes. Nucleic Acids Res. **28:** 2481–2493.

Verkade, H. M., and M. J. O'Connell, 1998 Cut5 is a component of the UV-responsive DNA damage checkpoint in fission yeast. Mol. Gen. Genet. **260:** 426–433.

Wang, H., and S. J. Elledge, 1999 DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **96:** 3824–3829.

Wang, Z., X. Wu and E. C. Friedberg, 1993 DNA repair synthesis during base excision repair in vitro is catalyzed by DNA polymerase epsilon and is influenced by DNA polymerases alpha and delta in Saccharomyces cerevisiae. Mol. Cell. Biol. **13:** 1051–1058.

Yamamoto, R. R., J. M. Axton, Y. Yamamoto, R. D. Saunders, D. M. Glover *et al.*, 2000 The Drosophila mus101 gene, which links DNA repair, replication and condensation of heterochromatin in mitosis, encodes a protein with seven BRCA1 C-terminus domains. Genetics **156:** 711–721.

Yamane, K., and T. Tsuruo, 1999 Conserved BRCT regions of TopBP1 and of the tumor suppressor BRCA1 bind strand breaks and termini of DNA. Oncogene **18:** 5194–5203.

Yamane, K., M. Kawabata and T. Tsuruo, 1997 A DNA-topoisomerase-II-binding protein with eight repeating regions similar to DNA-repair enzymes and to a cell-cycle regulator. Eur. J. Biochem. **250:** 794–799.

Zhang, X., S. Morera, P. A. Bates, P. C. Whitehead, A. I. Coffer *et al.*, 1998 Structure of an XRCC1 BRCT domain: a new protein-protein interaction module. EMBO J. **17:** 6404–6411.

Communicating editor: A. P. Mitchell