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CDC7/DBF4 Functions in the Translesion Synthesis Branch of the *RAD6* Epistasis Group in *Saccharomyces cerevisiae*

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ABSTRACT

CDC7 and *DBF4* encode the essential Cdc7-Dbf4 protein kinase required for DNA replication in eukaryotes from yeast to human. Cdc7-Dbf4 is also required for DNA damage-induced mutagenesis, one of several postreplicational DNA damage tolerance mechanisms mediated by the *RAD6* epistasis group. Several genes have been determined to function in separate branches within this group, including *RAD5*, *REV3/REV7* (Pol ζ), *RAD30* (Pol η), and *POL30* (PCNA). An extensive genetic analysis of the interactions between *CDC7* and *REV3*, *RAD30*, *RAD5*, or *POL30* in response to DNA damage was done to determine its role in the *RAD6* pathway. *CDC7*, *RAD5*, *POL30*, and *RAD30* were found to constitute four separate branches of the *RAD6* epistasis group in response to UV and MMS exposure. *CDC7* is also shown to function separately from *REV3* in response to MMS. However, they belong in the same pathway in response to UV. We propose that the Cdc7-Dbf4 kinase associates with components of the translesion synthesis pathway and that this interaction is dependent upon the type of DNA damage. Finally, activation of the DNA damage checkpoint and the resulting cell cycle delay is intact in *cdc7Δ mcm5-bob1* cells, suggesting a direct role for *CDC7* in DNA repair/damage tolerance.

ONE of the most important aspects of a cell's life cycle is the accurate replication, segregation, and structural maintenance of its genome. *Saccharomyces cerevisiae CDC7* encodes the catalytic subunit of a protein kinase that is involved in two of these processes, namely DNA replication and DNA repair (reviewed in Sclafani 2000). In DNA replication, Cdc7 protein is an essential regulator of this process and is thought to control initiation of replication by phosphorylating the Mcm2 protein, thereby activating the MCM helicase complex (reviewed in Bell and Dutta 2002). The requirement of Cdc7 in DNA repair was first suggested by the observation that the *cdc7-1* mutant is defective in induced mutagenesis when treated with different DNA-damaging agents, including UV light, methyl methanesulfonate (MMS), and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; Njagi and Kilbey 1982; Kilbey 1986). Furthermore, it has been determined that overexpression of *CDC7* causes an increase in induced mutation frequency (Sclafani *et al.* 1988), and both hyper- and hypomutagenic alleles of *cdc7* have been identified (Hollingsworth *et al.* 1992). On the basis of its DNA damage-induced mutagenesis phenotype and UV survival epistasis analysis, *CDC7* has been assigned to the *RAD6* epistasis group of DNA repair genes in *S. cerevisiae* (Njagi and Kilbey 1982).

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The *RAD6* epistasis group controls a poorly understood DNA repair pathway composed of several genes that, when mutated, result in sensitivity to a variety of DNA-damaging agents and, in many cases, also cause defects in damage-induced mutagenesis. At the core of this epistasis group is the Rad6 protein, a ubiquitin-conjugating enzyme that is required for all DNA damage tolerance processes (reviewed in Friedberg *et al.* 1995). Rad6 interacts with Rad18 protein, and it is thought that this complex is recruited to sites of DNA damage by the single-stranded DNA-binding activity of Rad18. Once there, the Rad6-Rad18 complex mediates DNA damage tolerance mechanisms by modifying the replication fork via its ubiquitin-conjugating activity, by protein degradation (Bailly *et al.* 1994), and/or as a signaling mechanism (Hofmann and Pickart 1999). It has been determined that the downstream components of this pathway are separated into more than one distinct branch, resulting in different mechanisms of DNA damage tolerance. Several models have been proposed for the genetic interactions between members of the *RAD6* epistasis group, namely *RAD5*, *MMS2*, *POL30*, *RAD30*, and *REV3*, and their roles in error-free or error-prone processes of DNA damage tolerance (McDonald *et al.* 1997; Ulrich and Jentsch 2000; Xiao *et al.* 2000).

The genes that are involved in error-free mechanisms of DNA damage tolerance, which are thought to occur via a DNA strand copy-choice process, include *RAD5*, *MMS2*, and *POL30* (reviewed in Broomfield *et al.* 2001). Mutations in these genes lead to a deficiency in postrep-

lication repair (PRR) activity following UV irradiation (Torres-Ramos *et al.* 1996, 2002), while showing little defect in DNA-damage-induced mutagenesis (Johnson *et al.* 1992; Torres-Ramos *et al.* 1996; Broomfield *et al.* 1998). *RAD5* encodes a 134-kD protein with a putative helicase domain and a cysteine-rich sequence motif (RING finger; Johnson *et al.* 1992). Also, Rad5 has been shown to form a homodimer and to mediate an interaction between the UBC13-MMS2 and RAD6-RAD18 complexes (Ulrich and Jentsch 2000). *MMS2* encodes a ubiquitin-conjugating enzyme variant protein that, in conjunction with the Ubc13 protein, forms a complex capable of assembling polyubiquitin chains linked through the K-63 residue of ubiquitin (Hofmann and Pickart 1999). Ubiquitin conjugation via K-63 is thought to have a specific signaling role in DNA damage tolerance, as a *UbK63R* mutation was shown to have DNA repair defects that fall within the *RAD6* epistasis group, while having no obvious impairment in protein degradation (Spence *et al.* 1995). *POL30* encodes proliferating cell nuclear antigen (PCNA), the processivity factor in eukaryotic DNA replication that is also involved in a variety of DNA repair processes, including nucleotide excision repair, base excision repair, and mismatch repair (Warbrick 2000). Mutational analysis of this gene identified the *pol30-46* allele, which shows increased sensitivity to DNA damage, but is normal for growth (Ayyagari *et al.* 1995). Genetic analysis of this mutant indicated that its DNA repair defects are specific to the *RAD6* epistasis group (Torres-Ramos *et al.* 1996) and that it functions in a branch separate from *RAD5* (Xiao *et al.* 2000). Because *pol30-46* strains show no defect in DNA-damage-induced mutagenesis, it was suggested that *POL30* is involved in error-free DNA damage tolerance (Torres-Ramos *et al.* 1996). However, it does not rule out the possibility that it might also play a role in error-prone mechanisms, and recent work has characterized a different allele of *POL30*, *pol30* (K164R), which is specifically defective in DNA-damage-induced mutagenesis and is epistatic to both *rev3*Δ and *rad30*Δ (Stelter and Ulrich 2003). This evidence suggests that PCNA is also involved in translesion synthesis (TLS) and that the *pol30-46* mutation knocks out a function of *POL30* specific to error-free processes of DNA damage tolerance.

The genes thought to mediate the error-prone process of DNA damage tolerance include *RAD30* and *REV3*. They both encode DNA translesion polymerases that are capable of replicating DNA past a damaged template (TLS; reviewed in Kunz *et al.* 2000; Prakash and Prakash 2002). *RAD30*, which encodes DNA polymerase η (Pol η), was identified in a search for homologs of the UmuC and DinB genes of *Escherichia coli* (McDonald *et al.* 1997; Roush *et al.* 1998). Deletion of *RAD30* results in increased sensitivity to UV and MMS exposure, and it was determined that *RAD30* constitutes a branch within the *RAD6* epistasis group that is separate from *RAD5* (McDonald *et al.* 1997; Roush *et al.* 1998). Pol η is remarkable for its accurate and efficient replication past a *cis-syn* thymine-thymine (T-T) dimer (Johnson *et al.* 1999b), and deficiencies in the human *RAD30* homolog were found to be responsible for the variant complementation group of xeroderma pigmentosum syndrome (Johnson *et al.* 1999a; Masutani *et al.* 1999b). Biochemical analysis has shown that Pol η has low processivity and low fidelity on undamaged template, but is capable of nucleotide insertion across a variety of DNA lesions with both mutagenic and nonmutagenic consequences (Minko *et al.* 2000; Washington *et al.* 2000; Yuan *et al.* 2000; Johnson *et al.* 2001); genetic evidence also indicates that Pol η TLS activity is required for bypass of a variety of DNA lesions and that it contributes toward MNNG-induced mutagenesis (Haracska *et al.* 2000; Bresson and Fuchs 2002).

REV3, which encodes the catalytic subunit of DNA polymerase ζ (Pol ζ; Morrison *et al.* 1989), was identified in a screen for mutants that resulted in a low frequency of UV-induced mutagenesis (Lemontt 1971). The Rev3 protein, together with the Rev7 protein, forms the heterodimeric Pol ζ, which was shown to be a translesion polymerase capable of bypassing a *cis-syn* (T-T) dimer (Nelson *et al.* 1996). Recently, biochemical characterization of Pol ζ revealed it to be a high-fidelity DNA polymerase that is very inefficient at bypassing template lesions (Johnson *et al.* 2000), but highly proficient at extending 3′ ends opposite DNA lesions (Guo *et al.* 2001; Haracska *et al.* 2001b, 2003). Genetic analysis, however, indicates that Pol ζ is required for the bypass of a variety of lesions, including a T-T pyrimidine (6-4) pyrimidone dimer [(6-4) T-T dimer; Baynton *et al.* 1998; Nelson *et al.* 2000; Lawrence 2002). In light of this evidence, the current model of TLS proposes that one or more DNA polymerases are required for this process, resulting in both mutagenic or nonmutagenic bypass, and that this is mainly a consequence of the type of lesion on the DNA template (Kunz *et al.* 2000; Broomfield *et al.* 2001; Prakash and Prakash 2002).

The role of *CDC7* in the *RAD6* pathway and within its error-free and error-prone branches is currently unknown. The studies done so far suggest that it plays a role in TLS mechanisms. However, given that the limited analysis of *CDC7* participation in DNA damage tolerance has focused on its defects in induced mutagenesis and has been carried out using only hypomorphic alleles, it does not rule out the possibility that it might also be required for replication restart in error-free processes. The isolation of the *mcm5-bob1* allele (Jackson *et al.* 1993; Hardy *et al.* 1997), which allows for the deletion of *CDC7*, provides us with a tool to carry out an extensive analysis of the genetic relationships between *CDC7* and other members of the *RAD6* epistasis group.

MATERIALS AND METHODS

Yeast strains, media, and plasmids: Yeast strains were grown in yeast extract/peptone/dextrose (YPD) with 2% glucose or in synthetic defined minimal media supplemented with

TABLE 1

Strains used in this study

Strains	Relevant genotype
299	*MAT*α *his3*Δ*1 leu2 trp1 ura3 can1 cyh2*
466	*MAT***a** *leu2 ura3 trp1 his7*
888	*MAT***a**/α *leu2*Δ*0/leu2*Δ*0 met15*Δ*0/+ ura3*Δ*0/ura3*Δ*0 his3*Δ*0/his3*Δ*0 +/lys2*Δ*0 rad5*Δ*::KanMX4/rad5*Δ*::KanMX4*
889	*MAT***a**/α *leu2*Δ*0/leu2*Δ*0 met15*Δ*0/+ ura3*Δ*0/ura3*Δ*0 his3*Δ*0/his3*Δ*0 +/lys2*Δ*0 rad30*Δ*::KanMX4/rad30*Δ*::KanMX4*
890	*MAT***a**/α *leu2*Δ*0/leu2*Δ*0 met15*Δ*0/+ ura3*Δ*0/ura3*Δ*0 his3*Δ*0/his3*Δ*0 +/lys2*Δ*0 rev3*Δ*::KanMX4/rev3*Δ*::KanMX4*
P211	*MAT***a** *ura3 lys2 cyh2 his3 leu2 cdc7*Δ*::HIS3 mcm5-bob1*
P235	*MAT***a** *ura3 lys2 cyh2 his3 leu2 cdc7*Δ*::HIS3 dbf4*Δ*::URA3 mcm5-bob1*
yLPB11	299 × P211
yLPB12	yLPB11 +*/rev3*Δ*::KanMX4*
yLPB14	yLPB11 +*/rad5*Δ*::KanMX4*
yLPB16	yLPB11 +*/rad30*Δ*::KanMX4*
yLPB18	*MAT***a** *ura3 his3 leu2 cyh2 trp1 mcm5-bob1*
yLPB21	*MAT*α *ura3 his3 leu2 cyh2 trp1 can1*
yLPB24	*MAT***a** *ura3 his3 leu2 cyh2 cdc7*Δ*::HIS3 mcm5-bob1*
yLPB25	*MAT*α *ura3 his3 leu2 cyh2 cdc7*Δ*::HIS3 mcm5-bob1*
yLPB26	*MAT*α *ura3 his3 leu2 cyh2 lys2 cdc7*Δ*::HIS3 mcm5-bob1*
yLPB27	*MAT*α *ura3 his3 leu2 cyh2 lys2 trp1 can1 rev3*Δ*::KanMX4*
yLPB28	*MAT***a** *ura3 his3 leu2 cyh2 trp1 rev3*Δ*::KanMX4*
yLPB29	*MAT***a** *ura3 his3 leu2 cyh2 can1 rev3*Δ*::KanMX4*
yLPB30	*MAT*α *ura3 his3 leu2 cyh2 lys2 trp1 can1 rad5*Δ*::KanMX4 mcm5-bob1*
yLPB31	*MAT***a** *ura3 his3 leu2 cyh2 lys2 trp1 can1 rad55*Δ*::KanMX4 mcm5-bob1*
yLPB32	*MAT*α *ura3 his3 leu2 cyh2 trp1 rad5*Δ*::KanMX4 mcm5-bob1*
yLPB34	*MAT***a** *ura3 his3 leu2 cyh2 trp1 can1 rad30*Δ*::KanMX4*
yLPB35	*MAT*α *ura3 his3 leu2 cyh2 trp1 can1 rad30*Δ*::KanMX4 mcm5-bob1*
yLPB37	*MAT***a** *ura3 his3 leu2 cyh2 trp1 can1 rev3*Δ*::KanMX4*
yLPB38	*MAT*α *ura3 his3 leu2 cyh2 lys2 trp1 rev3*Δ*::KanMX4*
yLPB42	*MAT*α *ura3 his3 leu2 cyh2 trp1 can1 rad30*Δ*::KanMX4*
yLPB45	*MAT*α *ura3 his3 leu2 cyh2 lys2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rev3*Δ*::KanMX4*
yLPB46	*MAT***a** *ura3 his3 leu2 cyh2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rev3*Δ*::KanMX4*
yLPB47	*MAT***a** *ura3 his3 leu2 cyh2 lys2 cdc7*Δ*::HIS3 mcm5-bob1 rev3*Δ*::KanMX4*
yLPB48	*MAT*α *ura3 his3 leu2 cyh2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rad5*Δ*::KanMX4*
yLPB49	*MAT*α *ura3 his3 leu2 cyh2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rad5*Δ*::KanMX4*
yLPB50	*MAT***a** *ura3 his3 leu2 cyh2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rad5*Δ*::KanMX4*
yLPB51	*MAT*α *ura3 his3 leu2 cyh2 lys2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rad30*Δ*::KanMX4*
yLPB52	*MAT***a** *ura3 his3 leu2 cyh2 can1 cdc7*Δ*::HIS3 mcm5-bob1 rad30*Δ*::KanMX4*
yLPB53	*MAT*α *ura3 his3 leu2 cyh2 lys2 cdc7*Δ*::HIS3 mcm5-bob1 rad30*Δ*::KanMX4*
yLPB54	yLPB11 +*/rev3*Δ*::KanMX4*
yLPB55	yLPB11 +*/rad5*Δ*::KanMX4*
yLPB62	*MAT***a** *ura3 his3 leu2 cyh2 trp1 mcm5-bob1 pol30-46*
yLPB63	*MAT*α *ura3 his3 leu2 cyh2 cdc7*Δ*::HIS3 mcm5-bob1 pol30-46*
yLPB65	*MAT*α *ura3 his3 leu2 cyh2 trp1 can1 pol30-46*
yLPB66	*MAT*α *ura3 his3 leu2 cyh2 trp1 can1 pol30-46*
yLPB74	*MAT*α *ura3 his3 leu2 cyh2 lys2 trp1 can1 rad5*Δ*::KanMX4 mcm5-bob1*::pRS306-MCM5
yLPB75	*MAT***a** *ura3 his3 leu2 cyh2 lys2 trp1 can1 rad5*Δ*::KanMX4 mcm5-bob1*::pRS306-MCM5
yLPB81	*MAT***a** *leu2 ura3 trp1 his7 RAD53::LEU2::3xHA-RAD53*
yLPB82	*MAT***a** *ura3 lys2 cyh2 his3 leu2 cdc7*Δ*::HIS3 mcm5-bob1 RAD53::LEU2::3xHA-RAD53*
yLPB83	*MAT***a** *ura3 lys2 cyh2 his3 leu2 cdc7*Δ*::HIS3 dbf4*Δ*::URA3 mcm5-bob1 RAD53::LEU2::3xHA-RAD53*
yLPB128	*MAT***a** *ura3 leu2 cyh2 his3 cdc7*Δ*::HIS3 mcm5-bob1 pol30-46*

appropriate amino acids and 2% glucose (Sclafani *et al.* 1988). All yeast strains used in this study are listed in Table 1. Strains 888, 889, and 890 were obtained from the Saccharomyces Genome Deletion Project and are in the S288c genetic background (Winzeler *et al.* 1999). All other strains are congenic with A364a (Hartwell 1967). Standard genetic methods were used for strain construction and tetrad analysis (Burke *et al.* 2000). Transformation of yeast strains was performed by the lithium acetate method (Ito *et al.* 1983).

*rev3*Δ::KanMX4, *rad5*Δ::KanMX4, and *rad30*Δ::KanMX4 disruption fragments were generated by PCR amplification of the gene locus using template genomic DNA from strains 888, 889, and 890. Genomic DNA was isolated as described (Lee 1992). The PCR primers used were REV3A, REV3D, RAD5A, RAD5D, RAD30A, and RAD30D from the Saccharomyces Genome Deletion Project.

*rev3*Δ, *rad5*Δ, and *rad30*Δ strains were generated by transforming strain yLPB11 with the respective gene disruption fragment, selecting for $G418^R$. Heterozygote diploids were identified by Southern genomic hybridization. Diploids were sporulated and dissected to generate haploid strains of the genotype desired. Gene disruptions were confirmed again by

TABLE 2

Plasmids used in this study

Plasmid	Genotype	Source/reference
pRS306-MCM5	Yip *URA3 MCM5*	This study
pLPB25	Yip *URA3 pol30-46*	This study
pLPB26	Yip *URA3 pol30-46*-NheI	This study
pLPB29	Yip *LEU2* 3×HA-*RAD53*	This study
pBL230-46	ARSH4 CEN6 *TRP1 pol30-46*	Ayyagari *et al.* (1995)
pPD61	ARSH4 CEN6 *URA3 RAD53*	Paul Dohrmann
pPD328	Yip *LEU2* 3×HA-rad53 (C terminus Δ)	Paul Dohrmann
pCH802	ARSH4 CEN6 *TRP1 CDC46*	Hardy *et al.* (1997)

Southern genomic hybridization. At least two independent isolates were generated for each genotype.

Due to the high recombination rate at the *MCM5* locus, proximal to the rDNA region on chromosome XII, it was important to determine the identity of the allele present, *MCM5* or *mcm5-bob1*, in the strains isolated. The original *mcm5-bob1* mutation (Hardy *et al.* 1997) ablates an *Eco*57I restriction site. This can be used as a diagnostic test on PCR fragments amplified using primers internal to the *MCM5* open reading frame, MCM5-Fwd (5′-CACCACTTCCTCCATTTCCACC-3′) and MCM5Rev (5′-CCCCAGATTTAGTGAATAAGAGCCC-3′). When no *MCM5* strains were isolated, *mcm5-bob1* strains were transformed with pRS306-MCM5, linearized with *Mlu*I, selecting for Ura$^+$. This generates a gene duplication, with one *MCM5* copy, which complements the *mcm5-bob1* mutation. Gene duplication was confirmed by Southern genomic hybridization.

All plasmids used in this study are listed in Table 2. Plasmid pRS306-MCM5 was constructed by cloning a 5.4-kb *Xho*I/*Not*I fragment from plasmid pCH802 into the *Xho*I/*Not*I sites of pRS306 (Sikorski and Hieter 1989). Plasmid pLPB25 was constructed by cloning a 1.1-kb *Bam*HI/*Sma*I fragment from plasmid pBL230-46 into the *Bam*HI/*Sma*I restriction sites in pRS306. Plasmid pLPB26, which introduces a unique *Nhe*I restriction site at codons eight and nine of the *POL30* open reading frame, was derived from pLPB25 using PCR-overlap mutagenesis (Ho *et al.* 1989). PCR was carried out using the outside primers M13Fwd (5′-TGTAAAACGACGGCCAGT-3′) and M13Rev (5′-TCACACAGGAAACAGCTATGAC-3′), complementary to the pRS306 backbone, and internal mutation primers POL30Nhe-Fwd (5′-GAAGAAGCtagCCTTTTCAAG-3′) and POL30Nhe-Rev (5′-CTTGAAAAGGctaGCTTCTTC-3′) (lowercase letters indicate silent mutations that introduce a *Nhe*I restriction site).

To obtain *pol30-46* strains, plasmid pLPB26 was linearized with *Nhe*I and transformed into *ura3*− strains yLPB18, yLPB21, and yLPB24, selecting for Ura$^+$. This results in a duplication of the *POL30* locus, with one of the copies being *pol30-46*. After growth in YPD, recombinant Ura$^-$ clones were selected for on SD − Ura + 5-fluoroorotic acid (5-FOA) media. Integration of the *pol30-46* allele was verified by PCR amplification and sequencing of the *POL30* locus. PCR amplification and sequencing were carried out using the POL30A and POL30D primers from the Saccharomyces Genome Deletion Project. *cdc7*Δ*::HIS3 mcm5-bob1 pol30-46* strains were also generated by mating strain yLPB26 with strain yLPB62. Diploids were sporulated and dissected, and double mutants were selected. The presence of the *pol30-46* mutation was followed by PCR amplification and sequencing, as stated above.

Plasmid pLPB29 was generated by cloning a 3.6-kb *Msc*I/*Xba*I insert from plasmid pPD61 into the 7.3-kb backbone fragment of plasmid pPD328. This generates a full-length 3× hemagglutinin(HA)-*RAD53* gene construct in a pRS305 (Sikorski and Hieter 1989) plasmid backbone. Plasmids pPD61 and pPD328 were a generous gift from Paul Dohrmann of this laboratory. Integration of plasmid pLPB29 at the *RAD53* locus was achieved by linearizing the plasmid with *Msc*I and transforming into *leu2*− strains selecting for Leu$^+$. This generates a *RAD53* duplication, with one copy tagged with three HA epitopes. Gene duplication was confirmed by Southern genomic hybridization.

DNA damage survival analysis: Cells were grown to mid-logarithmic phase (between 1 and 5×10^7 cells/ml) in YPD. Cell numbers were determined with a Coulter (Hialeah, FL) Multisizer II using a 100-μm orifice. For UV survival analysis, appropriate dilutions were plated in triplicate on YPD plates and were either untreated (0 J/m^2 control) or treated with specific UV doses with a 254-nm source at a fluency rate of 100 or 500 μW/cm^2, as measured with a UVP radiometer. Plates were incubated at 30° for 2–3 days, after which colonies were counted. UV exposure and plate incubation were carried out in the dark to avoid light-induced repair. The data presented represent the mean of at least three independent experiments. For MMS survival analysis, two different assays were carried out. For the first assay, 10-fold serial dilutions were spotted onto YPD plates either with no MMS for a control or with specific amounts of MMS added to it. All cultures were diluted to an initial concentration of 2×10^7 cells/ml (10° data point). For the second assay, 5×10^7 cells were resuspended in 5 ml of 0.1 M $NaPO_4$ buffer, pH 7.0, and treated with 0.5% MMS. Samples were removed before (0 min) and at different time points after addition of MMS. The samples were diluted sequentially into 10% sodium thiosulfate (to inactivate the MMS) and water and then plated on rich media to determine survival.

Mathematical analysis of UV survival curves: When the sensitivity of the double-mutant strain is greater than that of either single mutant, meaning that they are not epistatic, it is possible to determine the expected interaction between the two mutants if their relationship is additive using the natural logarithm of the surviving fraction ($-\ln S$) for each mutant (Brendel and Haynes 1973). This is given by the equation

$$-\ln S_{\text{double mutant}} = -\ln S_{\text{mutant 1}} + -\ln S_{\text{mutant 2}} - (-\ln S_{\text{WT}}).$$

If the observed $-\ln S_{\text{d.m.}}$ is greater than expected, as determined by the equation, one can conclude that the interaction between the two mutations is synergistic.

Fluorescence-activated cell sorter analysis: Cells were grown at 30° in 20 ml YPD to a density of 1–2 × 10^7 cells/ml. A sample for each culture was removed and processed for fluorescence-activated cell sorter analysis (FACS) as previously described (asynchronous time point; Ostroff and Sclafani 1995). The rest of the cultures were treated with synthetic α-factor at 10 μM

for 2 hr. Cell synchrony was monitored by phase-contrast microscopy at 400× magnification (90–95% unbudded cells indicated α-factor arrest). A sample for each culture was removed and processed for FACS (α-factor time point). The remainder of the cultures was split into two equal parts, washed to remove the α-factor, and resuspended in an equal volume of sterile water. One aliquot for each culture was exposed to 50 J/m^2 of UV, at a fluency rate of 1000 μW/cm^2, in a 100- × 15-mm petri plate with shaking to keep the cells in suspension; the second aliquot was treated equally, except for no UV exposure. The cultures were spun down, resuspended in 10 ml YPD, and incubated at 23°. UV exposure and subsequent incubation were carried out in the dark. Samples were removed from each culture every 20 min and processed for FACS.

Rad53 protein Western blot analysis: Strains with a 3× HA-Rad53 construct at its chromosomal locus were grown in 20 ml YPD to a density of ~2 × 10^7 cells/ml. Each culture was split into two aliquots, washed, and resuspended in 10 ml sterile water. For each culture, one aliquot was exposed to 100 J/m^2 of UV as described for FACS protocol; the second aliquot was a no UV control. After UV exposure, each aliquot was spun down, resuspended in 10 ml YPD, incubated at 23° for 40 min in the dark, and then processed for yeast protein extracts. Cells were washed in 2 ml PK lysis buffer [50 mM Tris (pH 7.6), 50 mM NaCl, 0.1% Triton X-100, 0.1% Tween 20, 1 mM EDTA] and then resuspended in 500 μl PK lysis buffer with 1.7 mg/ml phenylmethanesulfonyl fluoride (Sigma, St. Louis) and 500 μl 0.5-mm glass beads (Biospec Products, Bartlesville, OK) in 1.5-ml screw-cap tubes (Sarstedt, Newton, NC). Cells were lysed by agitation in a Mini-Beadbeater-8 (Biospec Products) with two 2-min bursts interspersed by 1 min on ice. The bottom of each tube was punctured by a needle, and the cell lysate was collected by spinning into a new Eppendorf tube. The lysates were spun at 14,000 × *g* for 15 min at 4° to remove insoluble material. An aliquot of the soluble protein was used to determine protein concentration by a BCA protein assay (Pierce, Rockford, IL). The remainder was combined with 5× SDS sample buffer (1× final) and boiled immediately for 5 min. Protein extracts (150 μg) were resolved by 7.5% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with 12CA5 anti-HA mouse monoclonal antibody (Boehringer Mannheim, Indianapolis) at 1:1000 dilution. Secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) was used at 1:3000 dilution. Immunoblots were visualized with an ECL chemiluminescence kit (Perkin-Elmer Life Sciences, Norwalk, CT).

RESULTS

***mcm5-bob1* has no effect on DNA damage survival:** To determine the role of *CDC7* within the *RAD6* pathway, we carried out a genetic analysis between *CDC7* and various members of the *RAD6* epistasis group. To avoid the problems that arise from using *cdc7* hypomorphic point mutations in this kind of analysis, we exploited the fact that the presence of the *mcm5-bob1* mutation permits the deletion of *CDC7*, which, otherwise, is an essential gene. Previous characterization of the *mcm5-bob1* mutation, compared to wild-type (WT) cells, indicated that it causes a slight decrease in the time it takes for yeast cells to enter the S phase of the cell cycle, but has no obvious impairment on the growth of the cells (Hardy *et al.* 1997). Since we planned to take advantage of the *mcm5-bob1* mutation to delete *CDC7*, we first examined what effect *mcm5-bob1* has on DNA damage survival. We found that *mcm5-bob1* cells are no more sensitive to UV irradiation than are WT cells (Figure 1), and the same is true for MMS exposure (data not shown). Comparison of a *rad5*Δ strain with a *rad5*Δ *mcm5-bob1* strain showed that the two strains exhibit the same degree of sensitivity to UV (Figure 1) and MMS exposure (data not shown), indicating that *mcm5-bob1* has no interaction with *rad5*Δ in response to DNA damage. This was also true for combinations of *mcm5-bob1* with *rad30*Δ, *rev3*Δ, or *pol30-46* (data not shown).

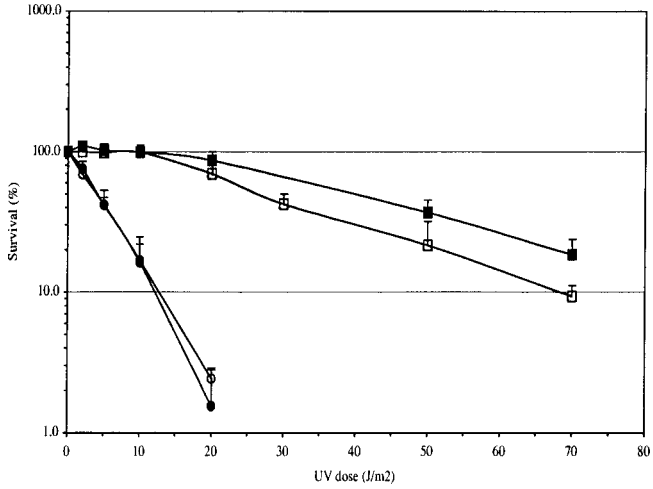

Figure 1.—*mcm5-bob1* has no effect on survival from UV irradiation. □, WT; ■, *mcm5-bob1*; ○, *rad5*Δ; ●, *rad5*Δ *mcm5-bob1*. Equal numbers of cells from logarithmically growing cultures were plated on YPD plates and irradiated with increasing doses of UV irradiation. Plates were incubated in the dark at 30° to determine viability.

We conclude that the *mcm5-bob1* mutation has no effect in the response to DNA damage exposure, and that we can use it as genetic tool to study the interaction between *cdc7*Δ and other mutations in the *RAD6* epistasis group. Given this, for simplicity, we omit reference to the *mcm5-bob1* allele whenever presenting data on *cdc7*Δ *mcm5-bob1* strains.

***CDC7* and *REV3* belong to the same pathway in response to UV treatment:** To determine which branch of the *RAD6* epistasis group *CDC7* belongs to, we carried out a UV survival epistasis analysis on strains combining *cdc7*Δ with a *rad5*Δ, *rad30*Δ, *rev3*Δ, or *pol30-46* mutation. Briefly, an equal number of cells for each of the strains were plated on YPD, after which they were exposed to different UV doses and incubated in the dark to determine cell survival. By comparing the phenotype of double mutations with that of the single mutant, it was determined that the *cdc7*Δ *rev3*Δ strain is no more sensitive than a *cdc7*Δ or *rev3*Δ strain alone, suggesting that *cdc7*Δ and *rev3*Δ are epistatic in response to UV damage (Figure 2A). In contrast, mathematical analysis (Table 3 and see materials and methods) of the single- and

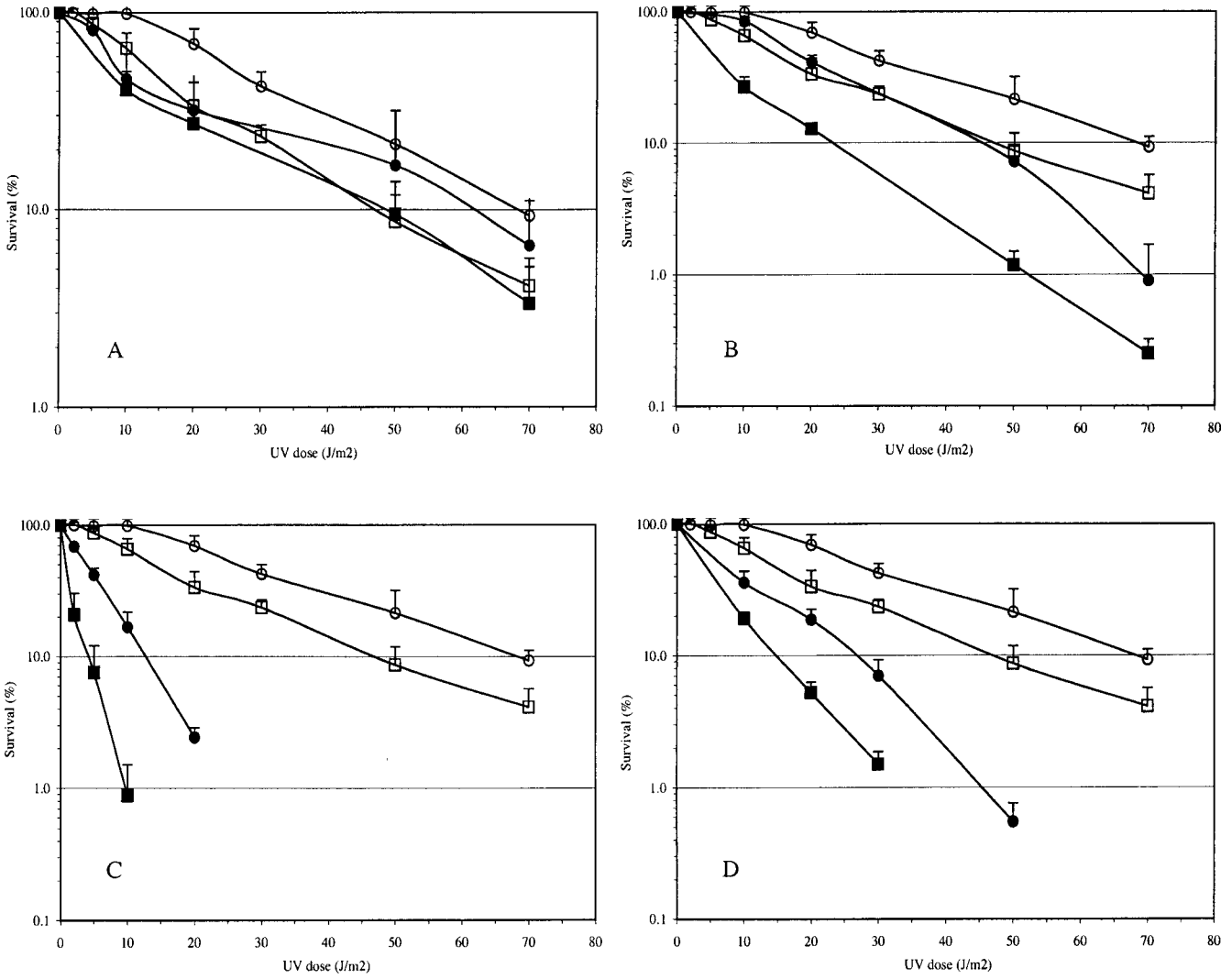

FIGURE 2.—*CDC7* and *REV3* are epistatic in response to UV exposure. (A–D) ○, WT; □, *cdc7*Δ. (A) *cdc7*Δ *vs. rev3*Δ. ●, *rev3*Δ; ■, *rev3*Δ *cdc7*Δ. (B) *cdc7*Δ *vs. rad30*Δ. ●, *rad30*Δ; ■, *rad30*Δ *cdc7*Δ. (C) *cdc7*Δ *vs. rad5*Δ. ●, *rad5*Δ; ■, *rad5*Δ *cdc7*Δ. (D) *cdc7*Δ *vs. pol30-46*. ●, *pol30-46*; ■, *pol30-46 cdc7*Δ. Equal numbers of cells from logarithmically growing cultures were plated on YPD plates and irradiated with increasing doses of UV irradiation. Plates were incubated in the dark at 30° to determine viability.

double-mutant survival data for *rad5*Δ in conjunction with *cdc7*Δ revealed that the *cdc7*Δ *rad5*Δ (Figure 2C) strain shows a synergistic response in UV sensitivity relative to the single-deletion strains in that the $-\ln S$ for the double-mutant ($-\ln S_{d.m.}$) strain is greater than expected for an additive interaction (BRENDEL and HAYNES 1973).

The analysis of survival data comparing the interaction between *cdc7*Δ and *rad30*Δ or *pol30-46* is not as straightforward (Table 3). While the observed $-\ln S_{d.m.}$ is greater than expected, the difference is not as large as when comparing *cdc7*Δ and *rad5*Δ. Furthermore, while for the *cdc7*Δ *rad5*Δ and *cdc7*Δ *pol30-46* double-mutant strains the difference between the observed and expected $-\ln S_{d.m.}$ increases with higher UV doses, this is not so for the *cdc7*Δ *rad30*Δ strain. Nevertheless, the data indicate that the double-mutant strains are more sensitive than either single-mutant strain and the interaction between *cdc7*Δ and *pol30-46* or *rad30*Δ is at least additive, if not synergistic. These results, together with previously published data, suggest that, in response to UV exposure, *CDC7*, *RAD5*, *POL30* (as indicated by the *pol30-46* allele), and *RAD30* constitute separate branches of the *RAD6* epistasis group.

***CDC7* represents a distinct *RAD6* branch in response to MMS treatment:** It has been determined that *CDC7* is required for DNA-damage-induced mutagenesis resulting from UV, MMS, MNNG, and EMS treatment (NJAGI and KILBEY 1982), whereas *REV3* is dispensable in MNNG (XIAO *et al.* 1999) and, possibly, EMS-induced (PRAKASH 1976) mutagenesis. This suggests that the requirement of *CDC7* and *REV3* for mutagenesis in response to different types of DNA-damaging agents is not always the same. Thus, we decided to investigate the genetic relationships between *CDC7* and the other members of the *RAD6* epistasis group in response to treatment with MMS.

To examine the interaction between *cdc7*Δ and *rad5*Δ,

TABLE 3

Mathematical analysis (Brendel and Haynes 1973) of UV survival data to determine if interaction between single-gene deletions is additive or higher

Strain	UV dose (J/m^2)	$-\ln S_{d.m}$[a] expected (if additive)	$-\ln S_{d.m.}$ observed
rad5Δ cdc7Δ	2	0.37	1.57
	5	0.98	2.59
	10	2.23	4.7
pol30-46 cdc7Δ	10	1.43	1.66
	20	2.4	2.96
	30	3.25	4.2
rad30Δ cdc7Δ	10	0.57	1.31
	20	1.62	2.06
	50	3.53	4.42
	70	5.5	5.81

[a] Surviving fraction for double-mutant strain (see materials and methods).

we used a qualitative serial dilution assay on rich media plates that contained specific amounts of MMS, compared to media with no MMS. The sensitivity of *cdc7Δ* in this assay is relatively mild and is notable only starting at MMS concentrations between 0.005 and 0.01% (data not shown). *rad5Δ* strains, on the other hand, are notably sensitive to MMS concentrations between 0.0005 and 0.001% (Figure 3 and data not shown). The double-mutant strain, however, shows a 5- to 10-fold increase in sensitivity with respect to *rad5Δ*, with notable killing at 0.0002% MMS (Figure 3). This shows that, as in response to UV, *cdc7Δ* and *rad5Δ* show a synergistic interaction upon MMS treatment.

The results for the plate assay examining the relationship between *cdc7Δ* and *pol30-46* were not as obvious. While the double mutant was more sensitive than either single mutant (data not shown), the difference was not as striking as above, which made interpretation of the results difficult. In light of this, we decided to carry out a quantitative assay, where we treated cells in suspension with 0.5% MMS for increasing periods of time, at which point an aliquot was removed, diluted, and plated on rich media to determine cell survival (Figure 4A). The same was done to examine the interaction between *cdc7Δ* and *rev3Δ* (Figure 4B) or *rad30Δ* (Figure 4C).

We find that the MMS sensitivity of the *pol30-46* strains generated here is not as strong as in previously published reports (Xiao *et al.* 2000; Broomfield and Xiao 2002). We note, however, that there are several differences between the strains used, including genetic background and the method used to introduce the *pol30-46* mutation (see materials and methods). Furthermore, differences in the MMS reagents used could be responsible for the discrepancy observed, as the UV sensitivity of the *pol30-46* strains is similar in both studies. When we examine the interaction between *cdc7Δ* and *pol30-46*, we find that the double-mutant strain is significantly more sensitive than either single mutant (Figure 4A). Analysis of the survival data (Table 4) indicates that this interaction is slightly stronger than additive at lower MMS doses (10 and 20 min), but no more so at a higher dose (30 min). Therefore, as observed above in response to UV damage, we conclude that the interaction between *cdc7Δ* and *pol30-46* in response to MMS treatment is at least additive.

Analysis of the MMS sensitivity of the *cdc7Δ rev3Δ* strain revealed a very different picture from what was observed in response to UV damage. In this case, the double mutant is significantly more sensitive than either single mutant, which exhibit very similar killing profiles in response to MMS exposure. The survival data for the double mutant (Table 4) also fluctuate between being slightly more and slightly less than what is expected of an additive effect. However, we conclude that *cdc7Δ* and *rev3Δ* exhibit an additive interaction.

Previous analyses of MMS sensitivity of a *rad30Δ* strain using a similar assay to the one used here have had conflicting outcomes; in one study, a *rad30Δ* strain is more sensitive than WT to MMS treatment (Roush *et al.* 1998), whereas in a second study the *rad30Δ* strain behaves no differently from a WT strain (Broomfield and Xiao 2002). We find that the *rad30Δ* strains generated in this report are as sensitive as WT to MMS killing. However, when combined with *cdc7Δ*, the double-mutant strain shows a significant increase in sensitivity compared to the *cdc7Δ* single mutant. This suggests that *RAD30* plays a minor role in the response to MMS treatment and that this role is separate from *CDC7*. In conclusion, our analysis of the interactions between *CDC7* and representative genes of distinct branches within the *RAD6* pathway indicates a distinct role for *CDC7* in response to MMS treatment.

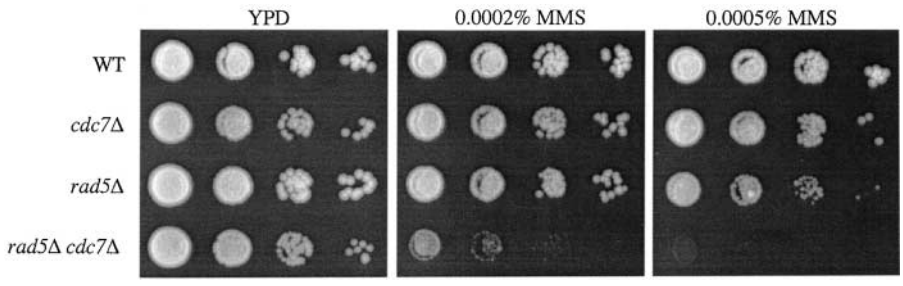

Figure 3.—Genetic interaction of *cdc7Δ* with *rad5Δ* in response to MMS treatment. Tenfold serial dilutions of logarithmically growing cultures were spotted, from left to right, onto YPD (control) or YPD plates with a specific concentration of MMS, as indicated.

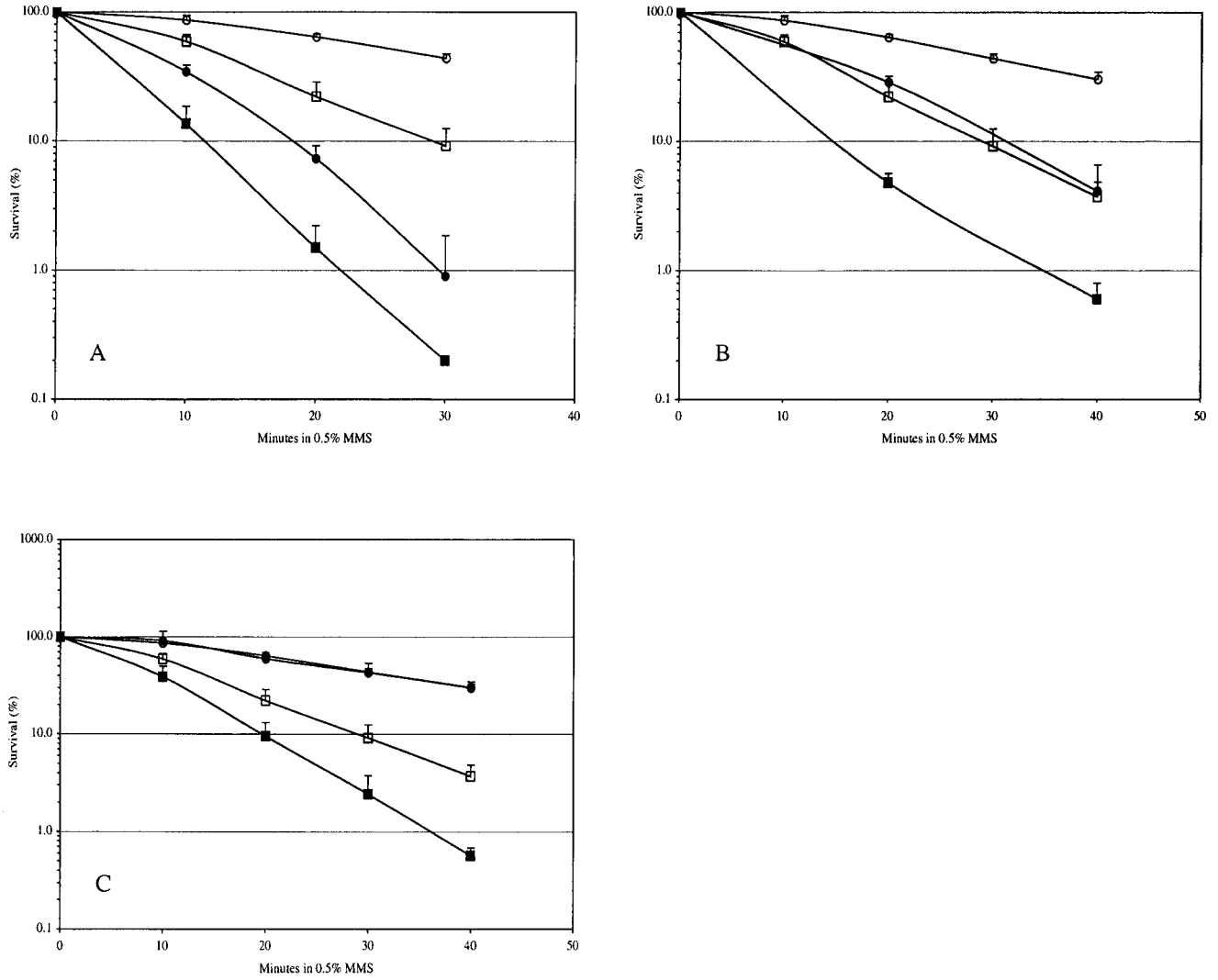

FIGURE 4.—Genetic interaction of *cdc7*Δ with *pol30-46*, *rev3*Δ, and with *rad30*Δ in response to MMS exposure. (A–C) ○, WT; □, *cdc7*Δ. (A) *cdc7*Δ *vs. pol30-46*. ●, *pol30-46*; ■, *pol30-46 cdc7*Δ. (B) *cdc7*Δ *vs. rev3*Δ. ●, *rev3*Δ; ■, *rev3*Δ *cdc7*Δ. (C) *rad30*Δ *vs. cdc7*Δ. ●, *rad30*Δ; ■, *pol30-46 cdc7*Δ. Cells in suspension were treated with 0.5% MMS for the amount of time indicated, at which point an aliquot was removed, diluted, and plated on YPD plates to determine viability.

The DNA damage checkpoint is intact in the absence of *CDC7*: One explanation for the phenotypes of *cdc7*Δ strains in response to DNA-damaging agents would be the possible role of the Cdc7/Dbf4 protein complex in checkpoint function. This aspect of *CDC7* function in genome maintenance is not well understood (JARES *et al.* 2000; SCLAFANI 2000). Initial studies with *cdc7*^{ts} mutants demonstrated that the DNA damage checkpoint was intact (SIEDE *et al.* 1994; OSTROFF and SCLAFANI 1995). However, because of the low sensitivity to UV light and possible leakiness of the hypomorphic alleles examined, we decided to reexamine the status of the DNA damage checkpoint in a *cdc7*Δ strain.

Previous analysis of a *rad9*Δ strain, which lacks a functional DNA damage checkpoint, showed that cells progress into S phase of the cell cycle independently of the presence of DNA damage. WT cells exposed to UV irradiation, on the other hand, showed a transient delay before progressing into S phase, when compared to unirradiated controls (SIEDE *et al.* 1993). We used a similar assay to determine if the G_1/S cell cycle delay caused by exposure to DNA-damaging agents is still present in a *cdc7*Δ strain. To do so, logarithmically growing cultures were first synchronized in G_1 using α-factor. The cultures were split into two aliquots and, immediately after release from the G_1 arrest, one aliquot was exposed to UV light. Then, at various time points, samples were collected for analysis of DNA content, allowing us to determine their progress through the cell cycle. When exposed to UV light, WT cells exhibited a delayed entry into S phase of the cell cycle. Cells that have not been exposed to UV light reach the G_2 phase of the cell cycle when UV-treated cells enter S phase (Figure 5A, compare WT + UV *vs.* −UV at 60 min). The same effect of UV exposure is observed for *cdc7*Δ cells (Figure 5B). It is not until 60 min after α-factor release that we begin

TABLE 4

Mathematical analysis (BRENDEL and HAYNES 1973) of MMS survival data to determine if interaction between single-gene deletions is additive or higher

Strain	0.5% MMS (min)	$-\ln S_{d.m.}$[a] expected (if additive)	$-\ln S_{d.m.}$ observed
rev3Δ cdc7Δ	20	2.32	3.04
	40	5.31	5.12
pol30-46 cdc7Δ	10	1.44	2.0
	20	3.69	4.2
	30	6.27	6.2

[a] Surviving fraction for double-mutant strain (see MATERIALS AND METHODS).

to see a shift in the DNA peak of UV-treated *cdc7Δ* cells, at which point the nontreated control is clearly progressing through S phase. After some time, irradiated *cdc7Δ* cultures overcome the cell cycle block and resume normal growth, eventually reaching stationary phase.

Second, we wanted to determine if the delay in cell cycle entry correlated with activation of the DNA damage checkpoint in response to UV exposure. To that end, we examined the phosphorylation status of the Rad53 protein, a key component of G_1/S, intra-S, and G_2/M checkpoints in *S. cerevisiae* (reviewed in NYBERG *et al.* 2002). We found that in WT or *cdc7Δ* cells that were exposed to UV light (Figure 5C) there was an upward shift of the Rad53 band migration, indicative of hyperphosphorylation of the protein and activation of the DNA damage checkpoint. Taken together, we conclude that the DNA damage checkpoint is intact and that the sensitivity of *cdc7Δ* cells to DNA-damaging agents is a result of the lack of Cdc7 function in DNA repair/damage tolerance mechanisms.

DISCUSSION

The role of *CDC7* in DNA damage tolerance is poorly understood. Previous data on induced mutagenesis and epistasis analysis indicated that *CDC7* belongs to the *RAD6* epistasis group, most likely within the TLS pathway. Accordingly, *CDC7*-mediated induced mutagenesis is restricted to the S phase of the cell cycle in agreement with its kinase activity profile (OSTROFF and SCLAFANI 1995; OSHIRO *et al.* 1999; WEINREICH and STILLMAN 1999). It is also known that different alleles of *cdc7* are either hyper- (*cdc7-3*, *-4*, *-23*) or hypomutagenic (*cdc7-1*, *-7*), even though they all exhibit reduced activity in DNA replication (HOLLINGSWORTH *et al.* 1992). Cdc7-Dbf4 kinase activity is required for mutagenesis, as a "kinase-dead" allele is defective in the process (HOLLINGSWORTH *et al.* 1992). This suggests a difference in affinity for downstream substrates of Cdc7 kinase in induced mutagenesis, although the identity of these is not known (SCLAFANI 2000). To gain a better understanding of the role of *CDC7* in DNA damage tolerance, we have carried out an extensive analysis of the genetic interactions between *CDC7* and members of the *RAD6* epistasis group.

The *mcm5-bob1* mutation does not affect *CDC7*-mediated DNA damage tolerance: To avoid the problems of

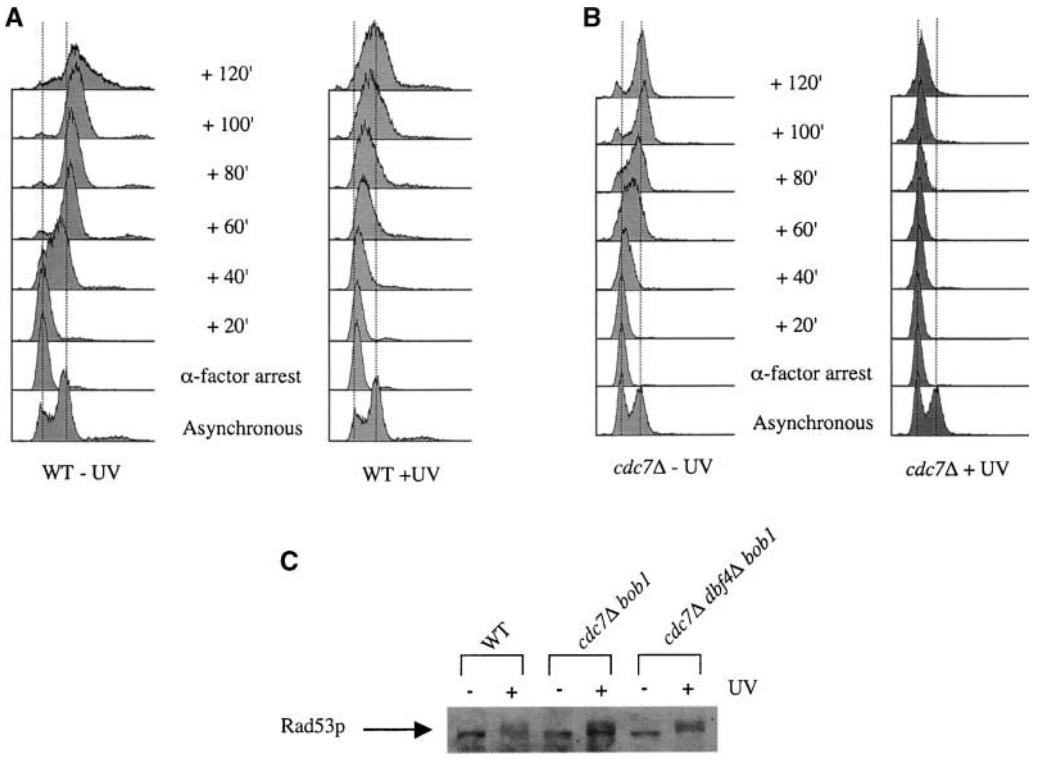

FIGURE 5.—The DNA damage checkpoint is intact in cells lacking *CDC7*. (A and B) Analysis of DNA content by FACS in (A) WT and (B) *cdc7Δ* cells. Cultures were synchronized in G_1 with α-factor, released, and immediately irradiated with UV light. Progression through the cell cycle was monitored by FACS. (C) Rad53 protein phosphorylation in response to UV treatment. Immunoblot of protein extracts isolated from logarithmically growing cells treated with + or − UV irradiation is shown.

using a *cdc7* hypomorphic allele in epistasis analysis, we took advantage of the fact that, in the presence of the *mcm5-bob1* mutation, we are able to delete *CDC7*. Our analysis of the *mcm5-bob1* mutation in response to DNA damage shows that it does not affect the sensitivity of yeast strains to UV or MMS, either by itself or in combination with other mutations in the *RAD6* epistasis pathway (Figure 1). Furthermore, previous work in our lab has also determined that *mcm5-bob1* has no effect on induced mutagenesis, either by itself or in combination with *cdc7*, suggesting that the bypass of *CDC7* is specific to DNA replication (Pahl 1994). Thus we have taken advantage of the *mcm5-bob1* mutation as a genetic tool to study the role of *CDC7* in DNA damage tolerance.

***CDC7* is specifically associated with error-prone mechanisms of DNA damage tolerance:** *RAD5* and *POL30* (as indicated by the *pol30-46* allele) represent two error-free pathways for DNA damage tolerance that are thought to rely on recombination/copy-choice mechanisms and are inherently nonmutagenic. Analysis of strains that combine *cdc7*Δ with *rad5*Δ, or *cdc7*Δ and *pol30-46*, revealed that they are more sensitive to UV irradiation and MMS exposure, compared to the single-mutant strains. The *cdc7*Δ *rad5*Δ strain showed a synergistic increase in sensitivity in response to the UV irradiation and MMS exposure, whereas the interaction between *cdc7*Δ and *pol30-46* gives only an additive decrease in cell survival.

The results of this genetic analysis, together with data in the literature (Ulrich and Jentsch 2000; Xiao *et al.* 2000), indicate that *RAD5*, *POL30*, and *CDC7* all function in separate pathways for DNA damage tolerance in *S. cerevisiae* (Figure 6). Furthermore, the synergism between *cdc7*Δ and *rad5*Δ (Table 3 and Figure 3) indicates that the two pathways compete for a common substrate resulting from DNA damage. The additive interaction between *cdc7*Δ and *pol30-46* (Tables 3 and 4) suggests that the affected pathways are independent from one another downstream of the point where they are blocked. However, it does not preclude the possibility that the initial substrate resulting from DNA damage is common (Cox and Game 1974). Finally, the data presented here infer that *CDC7* function is restricted to the TLS branch of DNA damage tolerance, an inherently error-prone mechanism that can result in the introduction of mutations, consistent with previous analyses (Njagi and Kilbey 1982; Hollingsworth *et al.* 1992).

The genetic interaction between *CDC7* and TLS polymerases is dependent on the nature of the DNA damage: The biochemical characterization of Pol η, encoded by the *RAD30* gene, and its role in xeroderma pigmentosum syndrome, suggest that it is a DNA polymerase specifically suited for the error-free bypass of *cis-syn* (T-T) dimers (Johnson *et al.* 1999a; Masutani *et al.* 1999a,b; Washington *et al.* 2000). However, it has been shown to contribute to the translesion of many DNA damage structures, including an O^6-methylguanine, and *N*-2-acetylaminofluorene modified guanine, although with lower efficiency (Haracska *et al.* 2000; Bresson and Fuchs 2002). These characteristics could account for the phenotypes of a *rad30*Δ strain, which shows a significant sensitivity to UV irradiation, but not other DNA damaging agents (this study; Roush *et al.* 1998; Haracska *et al.* 2000; Xiao *et al.* 2000; Broomfield and Xiao 2002).

Our analysis of the genetic interaction between *rad30*Δ and *cdc7*Δ indicates that the double mutant shows an additive or even slightly stronger increase in UV sensitivity (Table 3). This suggests that *RAD30* and *CDC7* function in separate pathways dealing with UV damage substrates and could reflect a specificity of the *RAD30* pathway for the bypass of *cis-syn* (T-T) dimers. The *CDC7*-mediated pathway, on the other hand, would deal primarily with other UV-induced damage structures. However, genetic studies have shown that *RAD30* is also involved in mutagenic bypass of a (6-4) T-T dimer (Bresson and Fuchs 2002). Given that *CDC7* is also required for UV-induced mutagenesis, it is possible that they function in separate pathways independently of the UV-induced substrate.

As mentioned above, the *rad30*Δ strain shows no increased sensitivity to MMS treatment, compared to a WT strain. However, the *cdc7*Δ *rad30*Δ strain is significantly more sensitive than a *cdc7*Δ strain to MMS. This suggests a very minor role of *RAD30* in response to MMS. The phenotype detected here is similar to the observation made in the study of the role of *RAD30* in MNNG-induced mutagenesis, which became apparent only when the *rad30*Δ was combined with a *pol32*Δ (Haracska *et al.* 2000). On the basis of our results, we propose that *CDC7* and *RAD30* function separately in response to MMS.

The analysis of a *cdc7*Δ *rev3*Δ strain indicates that *cdc7*Δ is epistatic to *rev3*Δ in response to UV irradiation (Figure 1), but shows an additive interaction in response to MMS treatment (Figure 3). This suggests that other cellular components contribute to the *CDC7* pathway within the *RAD6* epistasis group. One possibility is that Pol δ also contributes to *CDC7*-mediated DNA damage tolerance. *POL32*, a subunit of Pol δ, has been shown to be required for UV-, MMS-, and MNNG-induced mutagenesis, and *pol3-13*, a temperature-sensitive allele of the main subunit of Pol δ, is also defective in UV-induced mutagenesis. In addition, genetic analysis of *pol32*Δ and *pol3-13* determined that these two genes are in the same pathway as *REV3* (Giot *et al.* 1997; Haracska *et al.* 2000, 2001b; Huang *et al.* 2000) in response to UV damage. *pol32*Δ and *rev3*Δ have also been shown to be epistatic in response to MMS treatment. However, while *POL32* is required for MNNG-induced mutagenesis, *REV3* is not (Haracska *et al.* 2000; Huang *et al.* 2000).

The most striking observation from this study is the dependence of the genetic interactions between *cdc7*Δ and *rad30*Δ or *rev3*Δ on the type of DNA-damaging agent used. Most likely, this is a reflection of the variety of DNA damage structures that can arise from treatment with UV or MMS. As has been shown from *in vitro* and *in vivo* studies, Pol η (*RAD30*) and Pol ζ (*REV3/REV7*)

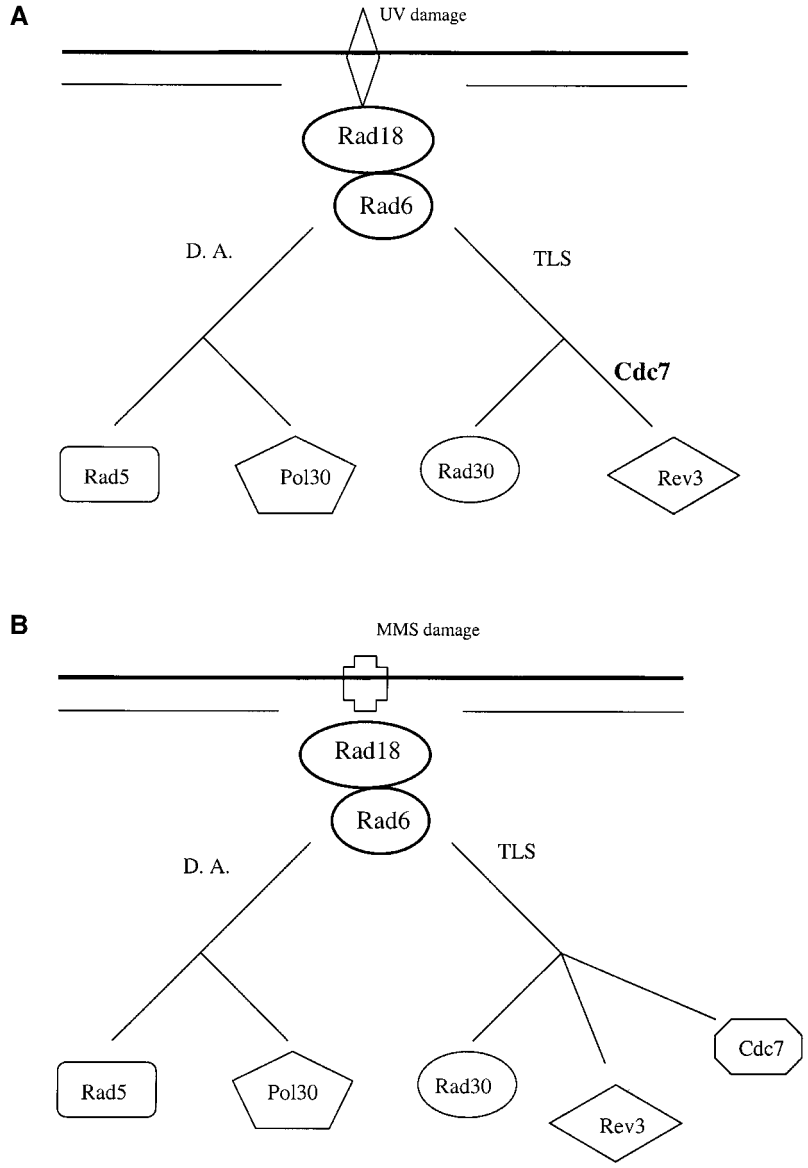

FIGURE 6.—Model for *CDC7* function in *RAD6*-mediated DNA damage tolerance in response to (A) UV and (B) MMS DNA damage. DNA damage is recognized by the Rad18/Rad6 protein complex and shuttled into different pathways for damage avoidance (D.A.) or translestion synthesis (TLS).

show marked differences in dealing with specific DNA damage structures. *In vitro* studies, however, do not necessarily reflect what is happening inside the cell. For example, the interaction between Rad30 and PCNA has been shown to be essential for the function of the polymerase *in vivo*, but this requirement is not seen in *in vitro* bypass assays of a *cis-syn* (T-T) dimer (HARACSKA *et al.* 2001a). Also, both *RAD30* and *REV3* are required for bypass of a (6-4) T-T dimer, and it has been proposed that they function together in this process (BRESSON and FUCHS 2002). However, the genetic analysis of *rad30*Δ and *rev3*Δ strains and the interaction between these two deletions do not support such a model (reviewed in LAWRENCE 2002).

The relationship between the many cellular components involved in TLS, as is understood now, is not very clear. The *in vivo* assays used to analyze the requirement of *RAD30* and *REV3* in the bypass of specific DNA damage structures can offer a strong insight into the mechanism of TLS. It seems clear that use of these assays to test the requirements of other TLS components, such as *REV1*, *POL32*, *POL30*, and now *CDC7* and/or *DBF4* will only add to our understanding of this important cellular process for dealing with the presence of DNA damage.

From the data presented here, we propose a model whereby the interaction between TLS components is dependent on the type of lesion encountered by the replication machinery. In the case of UV irradiation, or specific DNA damage structures resulting thereof, Cdc7 plays a role in the regulation of the Rev3/Rev7 pathway (Figure 6A). In other cases, such as alkylation damage, Cdc7 seems to be regulating a previously unidentified pathway (Figure 6B).

Activation of the DNA damage checkpoint in *S. cerevisiae* does not require *CDC7*: Finally, we address the idea

that Cdc7 is involved in the DNA damage checkpoint and the possible implications on the analysis of these results. Work in *Xenopus laevis* and *Schizosaccharomyces pombe* has shown that Cdc7 is important for checkpoint activation as a transducer and/or a target of checkpoint signaling (Jares *et al.* 2000; Snaith *et al.* 2000; Costanzo *et al.* 2003). Recently, it was shown that Cdc7/Dbf4 kinase is required for an etoposide-induced DNA damage checkpoint in the Xenopus system (Costanzo *et al.* 2003). Lack of checkpoint function is one explanation for the DNA damage sensitivity and mutagenesis phenotypes observed in *cdc7* mutants. To eliminate this possibility, we examined the status of the DNA damage checkpoint in *cdc7*Δ strains and found that both the G_1- to S-phase transition delay and the activation of Rad53 in response to UV exposure are intact (Figure 5). This is in agreement with experiments that show that the intraS-phase checkpoint is intact in *cdc7*Δ *mcm5-bob1* cells (Weinreich and Stillman 1999) and with recent work showing that *S. cerevisiae* Cdc7 is not required for checkpoint activation, maintenance, or downregulation in response to hydroxyurea (HU) or MMS treatment using *cdc7*^{ts} strains at the restrictive temperature (Tercero *et al.* 2003).

However, we cannot completely rule out the possibility that Cdc7 is a downstream target of the checkpoint. Dbf4 and Hsk1 (*Sp* Cdc7) are phosphorylated in a Rad53/Cds1 HU-treatment-dependent manner in *S. cerevisiae* and in *S. pombe*, respectively (Weinreich and Stillman 1999; Snaith *et al.* 2000). Furthermore, other checkpoint proteins have been shown to have a role in DNA damage-induced mutagenesis (Paulovich *et al.* 1998; Kai and Wang 2003). We recognize the possibility that the Cdc7 function in DNA damage tolerance is induced by checkpoint activation, but argue that this function is directly involved in the TLS mechanism.

What is the role of Cdc7 function in TLS? Previous data from our laboratory, and the work presented here, reveal a role for Cdc7 protein and its kinase activity in TLS. In DNA replication, Cdc7 may phosphorylate Mcm2 protein, a subunit of the hexameric MCM complex. The *mcm5-bob1* mutation bypasses the requirement of Cdc7 in DNA replication, but not in induced mutagenesis using *cdc7*^{ts}, *cdc7*Δ, and *cdc7*Δ *dbf4*Δ strains. In addition, *mcm5-bob1* on its own has no effect on cell survival or induced mutagenesis (Pahl 1994), and there is no evidence implicating the involvement of other MCM subunits in DNA damage tolerance. This suggests that the substrate for Cdc7-Dbf4 in TLS may be different from its substrate in DNA replication.

It is thought that TLS occurs via a DNA polymerase switch, whereby the replicative polymerase is substituted by another one capable of bypass, allowing the replication fork to progress through the damage (Kunz *et al.* 2000; Broomfield *et al.* 2001; Prakash and Prakash 2002). It has been proposed that, in some cases, this switch occurs more than once, as more than one polymerase might be required for efficient bypass. Although it is not known how this exchange occurs, or how it is regulated, it is reasonable to expect that it involves proteins already present at the replication fork and/or others that are brought to it when replication stalls. The target of Cdc7 phosphorylation is likely to be one of these—possibly the bypass polymerases themselves or an accessory protein, such as Pol32, Rev1, Rev7, or PCNA.

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