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The fission yeast Rad3 protein is representative of a class of proteins that play a crucial role in genome maintenance in all eukaryotic cell. In cells lacking Rad3, normal cell cycle arrest and DNA repair are not induced in response to damage. As a result, such cells are extremely sensitive to DNA damage or inhibition of DNA replication. The related human protein, Atm, is responsible for the severe congenital disorder Ataxia Telengiectasia, which is associated with radiation sensitivity and greatly elevated cancer risk. Related proteins have been found in many other eukaryotes including budding yeast, flies, frogs and mice, where they also play a crucial role in the response to DNA damage. All of these proteins are large (>200kD) protein kinases with a conserved kinase motif in the C-terminus. Although the kinase motif mostly closely resembles phosphoinositol-3 kinases, it is only known to phosphorylate proteins. Our working model is that damage selectively activates the Rad3 kinase, which then phosphorylates substrates involved in cell cycle control and repair of DNA damage. The goal of this project was to identify Rad3 interacting proteins in order to learn how Rad3 is activated and what its substrates are.

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TABLE OF CONTENTS

(5) INTRODUCTION

The fission yeast Rad3 protein is representative of a class of proteins that play a crucial role in genome maintenance in all eukaryotic cell. In cells lacking Rad3, normal cell cycle arrest and DNA repair are not induced in response to damage. As a result, such cells are extremely sensitive to DNA damage or inhibition of DNA replication. The related human protein, Atm, is responsible for the severe congenital disorder Ataxia Telengiectasia, which is associated with radiation sensitivity and greatly elevated cancer risk. Related proteins have been found in many other eukaryotes including budding yeast, flies, frogs and mice, where they also play a crucial role in the response to DNA damage. All of these proteins are large (>200kD) protein kinases with a conserved kinase motif in the C-terminus. Although the kinase motif mostly closely resembles phosphoinositol-3 kinases, it is only known to phosphorylate proteins. Our working model is that damage selectively activates the Rad3 kinase, which then phosphorylates substrates involved in cell cycle control and repair of DNA damage. The goal of this project was to identify Rad3 interacting proteins in order to learn how Rad3 is activated and what its substrates are.

(6) BODY

Development of reagents and methodologies. An initial goal was to develop reagents and methods that could be used to study Rad3 and interacting proteins. We developed a polyclonal antibody against Rad3, and were able to use it to detect Rad3 by western blot or immunoprecipitation when the protein was overexpressed from a plasmid (TO I, task 14). However we were never able to detect endogenous levels of Rad3 with this antibody. We also constructed HA-epitope-tagged versions of Rad3 and were again able to detect these proteins when they were moderately overexpressed by western blotting or immunoprecipitation (TO I, task 11). In addition, we developed an assay for Rad3 kinase activity using exogenously added PHAS-1 as a substrate. This is was not part of the initial statement of work, but is consistent with the general objectives of the grant.

Screens for Rad3-interacting proteins. We used a variety of approaches in unsuccessful attempts to identify Rad3-interacting proteins. We conducted an extensive screen for high-copy supressors of *rad3* mutants (TO I.i). This screen did not yield any convincing suppressors. We believe this may be because we used rad3 alleles from a genetic screen done in 1989. None of these alleles have been characterized at the molecular level and it is possible that they are not good choices for this

screen. We therefore decided to revise our strategy and begin by generating well characterized alleles of *rad3* which could then be used for a more directed supressor screen.

We also conducted an extensive 2-hybrid screen (this was not in the original proposal, but is consistent with its objectives). This also did not lead to the identification of any interacting proteins. We have heard from others that this approach also did not work for Atm or for the *S. cerevisiae* protein, Mec1. We decided that the 2-hybrid approach might have more chance for success if we could use a smaller piece or Rad3 that might be interact more efficiently with substrates or co-activators.

Functional analysis of Rad3 sequences. Given the difficulties we were experiencing with the genetic screen, we decided to revise our strategy for identifying Rad3-interacting proteins. We reasoned that since Rad3 is a large protein, it is likely to contain many protein-protein interaction motifs. We decided to search for small sequences that were required for Rad3 function in order to identify such motifs. Ultimately this will allow us to use smaller pieces of Rad3 as "baits" in the 2-hybrid screen, thus increasing the likelihood of its success. It will also enable enable us to design and construct *rad3* alleles with very selective deficits, which could be used to search for high-copy suppressors (TOI.i).

We first investigated the role of sequences outside the conserved kinase domain in Rad3 function. A previous study of ATM had suggested that the kinase domain is necessary and sufficient for ATM function. We constructed a series of *rad3* alleles with progressively larger deletions of the N-terminal region. The alleles were assayed for complementation of HU- an UV sensitivity of *rad3* null strains, and for *in vitro* kinase activity. None of the mutant proteins were catalytically active, and none were able to complement *rad3* mutants, indicating that, in contrast to ATM, the Rad3 N-terminal sequences are essential for Rad3 function.

We next developed methods for further defining essential Rad3 sequences outside the kinase domain. We found that modest overexpression of a protein consisting of the N-terminal 775 amino acids of Rad3 disrupted checkpoint control in wild-type cells. In contrast, checkpoint control was normal in cells overexpressing the C-terminus or Rad3, including the kinase domain. We reasoned that the N-terminal fragment is acting as a dominant negative because it competes with endogenous Rad3 for binding to co-activators or substrates. To pinpoint the sequences in the N-terminus that are the cause of dominant-negative activity, we constructed a series of deletion mutants and assayed them for dominant negative activity. This

analysis revealed that two motifs are required for dominant-negative activity: a sequence with similarity to leucine zippers and a sequence with similarity to PCNA-binding motifs found in proteins such as Rad21 and p21. We next examined the ability of full-length Rad3 proteins lacking either of these motifs to complement rad3 null alleles and to phosphorylate substrates in vitro. Neither deletion allele was able to complement the rad3 null, indicating that the sequences we had identified using the dominant negative assay were required for function of the full-length protein. However both mutants were as catalytically active as wild-type proteins indicating that the motifs are not required for kinase activity, but maybe required for interaction with substrates or regulators.

Chk1 has previously been shown to associate with Rad3, and Rad3 is also known to homodimerize. We used the assays we had developed in task 11 to show that neither of the mutants we had constructed was disrupted for dimerization or interaction with Chk1.

These studies were published as a full-length research *in Molecular Biology of the Cell* (see Appendix I), and comprised a chapter of Carolyn Riley Chapman's thesis which was successfully defended on July 30, 1999. Others in the lab are currently using the alleles she generated in supressor screens and in redesigned 2-hybrid screens.

Genetic interactions linking *rad3+* to DNA replication. We had initially planned to use classical genetic techniques to search for rad3 suppressors (TO I. ii, Task 6). However given the problems we had finding high-copy suppressors using the *rad3* alleles we had, we decided to use a different strategy. Kroll et al (*Genetics* 143: 95-102) reported on a type of genetic interaction called "synthetic dosage lethality". This describes disruption of a protein complex by combining overexpression of one subunit with a weak loss-of-function mutation in another subunit. We reasoned that we could use this strategy to genetically identify components of the Rad3 complex by looking for mutations that had no phenotypes on their own, but were lethal when examined in combination with Rad3 overexpression.

We used this approach to test for interactions between Rad3 and components of the DNA replication machinery. These were chosen for analysis because Rad3 is activated by unreplicated DNA and also regulates DNA progression in response to DNA damage. It is therefore likely that DNA replication enzymes are co-activators and/or substrates of Rad3.

We tested a number of replication mutants, and were able to observe a striking interactions between Rad3 overexpression and specific alleles of DNA polymerase d. Specifically Rad3 overexpression significant lowered

the restrictive temperature of these mutants and caused them to undergo cell cycle arrest. We used physiological and genetic techniques to show that the arrest is likely to be due to problems completing DNA replication.

These experiments comprised a chapter in Carolyn Riley Chapman's thesis which was defended on July 30, 1999. We are in the process of preparing a manuscript describing them for submission to *Genetics*.

SUMMARY AND CONCLUSION

The analysis of Rad3 did not proceed as we anticipated in our initial research proposal. Specifically we were unable to identify interacting protein using either high-copy suppressors or the 2-hybrid screen. As a result we had to revise or replace many of the technical objectives and tasks outlined in our original SOW. Using the revised tasks we were able to learn important new things about Rad3 function as a result of this research. Specifically, we were able to identify to Rad3 motifs that are likely to be involved in protein-protein interactions, and we have obtained evidence suggestive of an interaction between Rad3 and replication proteins. These studies are an excellent foundation for the study of Rad3 that will form the basis of future studies to be conducted in my lab.

(7)

1) Key research accomplishments.

- Preparation of polyclonal antibodies to Rad3.
- Construction of wild-type an mutant *rad3* alleles
- Development of a Rad3 kinase assay
- Identification of 2 motifs, the leucine-zipper and the P-site, which are required for Rad3 function.
- Demonstration of genetic interaction between rad3 and components of the DNA replication complex.

2) Reportable outcomes.

Manuscripts:

Chapman, C. R., Evans, S. T., Carr, A. M., and Enoch, T. (1999). Requirement of sequences outside the conserved kinase domain of fission yeast Rad3p for checkpoint control. *Mol. Biol. Cell* 10: 3223-3238.

Chapman, C. R. and Enoch, T. (2000) Genetic interactions link fission yeast *rad3*+ to DNA replication. *in preparation*..

Abstracts:

Evans, S. T., Chapman, C. R. and Enoch, T. (1999) Isolation of new alleles of Rad3 and Cds1 kinases in Schizosaccharomyces pombe. abstract presented at the First International Fission Yeast Meeting, Edinburgh, Scotland, U. K. September, 1999.

Degrees:

Doctor of Philosophy in Genetics awarded to Carolyn Riley Chapman in September of 1999.

Funding:

"The Role of Cds1 in Recovery from S-phase Arrest in Fission Yeast". Predoctoral Fellowship awarded to Sarah T. Evans to explore observations made in collaboration with Carolyn Riley Chapman.
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Dr. Chapman was hired by L. E. K. Consulting Inc. in September, 1999.

APPENDIX I.

Evans, S. T., Chapman, C. R. and Enoch, T. (1999) Isolation of new alleles of Rad3 and Cds1 kinases in Schizosaccharomyces pombe. abstract presented at the First International Fission Yeast Meeting, Edinburgh, Scotland, U. K. September, 1999.

Isolation of new alleles of the Rad3 and Cds1 kinases in *Schizosaccharomyces pombe*

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Cells activate a number of pathways in response to blocked DNA replication caused by the drug hydroxyurea (HU). Among them are a pathway that leads to mitotic arrest and one which permits "recovery" from this arrest. Mitotic arrest ensures that the cells do not divide with unreplicated DNA while the "recovery" response permits the cells to successfully exit from S phase arrest. The signal transduction pathways which sense incomplete DNA replication and transmit signals to carry out these responses contain several important protein kinases.

The Rad3 kinase, a homolog of *S. cerevisiae* Mec1 and human Atr and Atm, is thought to be involved in both of these responses. The Rad3 protein is a somewhat unusual protein kinase. Though its kinase domain exhibits significant homology to lipid kinases, it like the other members of this phosphatidylinositol-3 kinase related kinase (PI3KR) family is thought to phosphorylate only proteins, not lipids. In agreement with previous studies, we have found that Rad3, like the related Atm and Atr kinases, has protein kinase activity *in vitro*. However, constructs consisting of only the kinase domain do not fully complement either the hydroxyurea (HU) or the ultraviolet light (UV) sensitivity of *rad3*$^-$ mutants and do not have kinase activity *in vitro*, establishing that sequences in the N-terminus, outside the conserved kinase domain, are required for Rad3 function. We have also used this assay to identify alleles of Rad3 which retain kinase activity but cannot complement the HU sensitivity of a *rad3Δ* strain. Thus these alleles are defective in a novel function of Rad3, perhaps in the binding of substrates or regulators.

The Cds1 kinase, a substrate of the Rad3 kinase, appears to play a particularly important role in the recovery pathway as *cds1*$^-$ mutants are competent in the mitotic arrest pathway and specifically defective in the recovery pathway. Using PCR mutagenesis followed by gap repair, we have generated several temperature and cold sensitive alleles of *cds1*$^-$ with the hope that these partial loss of function alleles will be useful in a suppressor screen to isolate additional members of the recovery pathway. Interestingly, several of these alleles are mutated in the N-terminal forkhead associated (FHA) domain, demonstrating that the FHA domain is required *in vivo* for Cds1 function. Structure/function analysis of these alleles is also underway.

APPENDIX II.

Chapman, C. R., Evans, S. T., Carr, A. M., and Enoch, T. (1999). Requirement of sequences outside the conserved kinase domain of fission yeast Rad3p for checkpoint control. *Mol. Biol. Cell* 10: 3223-3238.

Molecular Biology of the Cell
Vol. 10, 3223–3238, October 1999

Requirement of Sequences outside the Conserved Kinase Domain of Fission Yeast Rad3p for Checkpoint Control

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The fission yeast Rad3p checkpoint protein is a member of the phosphatidylinositol 3-kinase-related family of protein kinases, which includes human ATMp. Mutation of the *ATM* gene is responsible for the disease ataxia-telangiectasia. The kinase domain of Rad3p has previously been shown to be essential for function. Here, we show that although this domain is necessary, it is not sufficient, because the isolated kinase domain does not have kinase activity in vitro and cannot complement a *rad3* deletion strain. Using dominant negative alleles of *rad3*, we have identified two sites N-terminal to the conserved kinase domain that are essential for Rad3p function. One of these sites is the putative leucine zipper, which is conserved in other phosphatidylinositol 3-kinase-related family members. The other is a novel motif, which may also mediate Rad3p protein–protein interactions.

INTRODUCTION

Checkpoint pathways ensure the correct temporal order of the cell cycle (Hartwell and Weinert, 1989) and are an evolutionarily conserved feature of eukaryotic cells. The *Schizosaccharomyces pombe* checkpoint kinase Rad3p (unrelated to the *Saccharomyces cerevisiae* DNA helicase Rad3p) acts to delay cell cycle events in response to DNA damage or incomplete DNA replication (Jimenez *et al.*, 1992; Seaton *et al.*, 1992; Bentley *et al.*, 1996). Unlike wild-type cells, *rad3*Δ cells do not arrest the cell cycle in response to DNA damage or blocked DNA replication and lose viability rapidly under these conditions (Al-Khodairy and Carr, 1992). Two human proteins with similarities to fission yeast Rad3p, ATRp (Bentley *et al.*, 1996; Cimprich *et al.*, 1996) and ATMp (Savitsky *et al.*, 1995a,b), also function in checkpoint control (Painter and Young, 1980; Beamish and Lavin, 1994; Cliby *et al.*, 1998). In humans, mutation of the *ATM* gene causes the autosomal recessive disease ataxia-telangiectasia (A-T), in which patients suffer from a variety of symptoms, including a predisposition to cancer (Harnden, 1994). In mice, deletion of the *ATM* gene greatly increases the frequency of thymic lymphomas (Barlow *et al.*, 1996; Xu *et al.*, 1996). Thus, normal checkpoints appear to be required to prevent cancer in mammalian cells. Fission yeast provides the opportunity to study these checkpoint kinases in a simple, genetically tractable model system.

Fission yeast Rad3p and human ATMp and ATRp are members of a large family of structurally and functionally similar proteins from diverse organisms (Savitsky *et al.*, 1995a; Bentley *et al.*, 1996, Cimprich *et al.*, 1996). Other members of the family include human DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase (Hartley *et al.*, 1995), and FKBP12–rapamycin-associated protein (Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994); *S. cerevisiae* Mec1p (Weinert, 1992; Kato and Ogawa, 1994), Tel1p (Greenwell *et al.*, 1995; Morrow *et al.*, 1995), Tor1p, and Tor2p (Heitman *et al.*, 1991; Kunz *et al.*, 1993); and *Drosophila* Mei-41p (Hari *et al.*, 1995). *S. pombe* Tel1p has also recently been identified (Naito *et al.*, 1998). At their C termini, these large proteins (>200 kDa) all contain a kinase domain related to phosphatidylinositol 3-kinases (PI3Ks). Despite this similarity, none of the PI3-kinase-related (PI3KR) proteins have been shown to phosphorylate lipids. ATMp, ATRp, DNA-PK, and Rad3p are all capable of autophosphorylation (Bentley *et al.*, 1996; Chan and Lees-Miller, 1996; Cliby *et al.*, 1998; Scott *et al.*, 1998), and are also known to directly phosphorylate other protein substrates as well (Lees-Miller *et al.*, 1992; Banin *et al.*, 1998; Canman *et al.*, 1998, Martinho

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Abbreviations used: A-T, ataxia-telangiectasia; FACS, fluorescence-activated cell-sorting; HA, hemagglutinin; HU, hydroxyurea; LZ, leucine zipper; PCNA, proliferating cell nuclear antigen; PI3KR, phosphatidylinositol 3-kinase-related; TBS, Tris-buffered saline; TBST, TBS and Tween 20.

et al., 1998). In addition to the kinase domain, many PI3KR proteins contain a putative leucine zipper motif, which may mediate homo- or heterodimerization.

The PI3KR family can be subdivided on the basis of sequence similarity (Keith and Schreiber, 1995; Bentley *et al.*, 1996). In such a phylogenetic tree, *S. pombe* Rad3p, human ATRp, *Drosophila* Mei-41p, and *S. cerevisiae* Mec1p form one subgroup (the ATR group), whereas human ATMp, *S. cerevisiae* Tel1p, and *S. pombe* Tel1p are in another distinct but closely related cluster (the ATM group). In yeast and in higher eukaryotes, ATR and ATM-like proteins appear to have overlapping molecular functions in cell cycle control and telomere regulation. For example, *S. cerevisiae mec1 tel1* and *S. pombe rad3 tel1* double mutants have more severe defects than either single mutant (Morrow *et al.*, 1995; Naito *et al.*, 1998). Moreover, overexpression of *S. cerevisiae tel1*$^+$ partially complements the radiation sensitivity of *mec1* mutants (Morrow *et al.*, 1995), and human *ATR* overexpression can suppress A-T cell defects (Cliby *et al.*, 1998). In contrast, the remaining family members are more distantly related and have not been shown to overlap functionally with the ATM and ATR subgroups. *S. cerevisiae* Tor1p and Tor2p and human FKBP12–rapamycin-associated protein regulate G1 progression, whereas DNA-PKcs is involved in V(D)J recombination and repair of double-stranded DNA breaks.

Although not directly involved in checkpoint control, DNA-PKcs serves as a paradigm for understanding the regulation of other kinases in the family because the biochemistry of its regulation is relatively well understood (Jeggo *et al.*, 1995). In vitro, DNA-PKcs is activated by binding double-stranded DNA ends in the presence of a heterodimer, composed of Ku70 and Ku86 subunits (Lieber *et al.*, 1997). In this way, the activity of this kinase is dependent both on specific DNA structures and on regulatory cofactors.

Analogously, the ATR and ATM-like PI3KR kinases may also require regulatory cofactors and may be activated by DNA structures. Although the activating structures and regulatory cofactors for these kinases have not been identified, it is known that ionizing radiation enhances the ATM-dependent phosphorylation of p53 in mammalian cells (Banin *et al.*, 1998; Canman *et al.*, 1998). Similarly, a number of fission yeast proteins, including Hus1p, Chk1p, and Cds1p, are phosphorylated in response to DNA damage in a Rad3p-dependent manner (Walworth and Bernards, 1996; Kostrub *et al.*, 1998; Lindsay *et al.*, 1998), suggesting that Rad3p activity could also be activated by DNA damage. The phosphorylation of these proteins is also dependent on other fission yeast checkpoint proteins, which do not contain recognizable kinase domains, such as Rad1p, Rad9p, Rad17p, and Rad26p (Sunnerhagen *et al.*, 1990; Enoch *et al.*, 1992; Rowley *et al.*, 1992; Al-Khodairy *et al.*, 1994). These proteins, as well as others known to be involved in checkpoint control (McFarlane *et al.*, 1997; Saka *et al.*, 1997; Willson *et al.*, 1997), are candidate cofactors or regulators of Rad3p. For example, they may stimulate Rad3p kinase activity by directing the localization of Rad3p to activating DNA structures such as sites of DNA damage, just as the Ku proteins regulate DNA-PKcs.

The Rad3p kinase domain, which comprises <15% of the protein, is essential for Rad3p function in cell cycle checkpoint control (Bentley *et al.*, 1996). Little is known about the function of Rad3p sequences outside the kinase domain. In this study we demonstrate the importance of sequences outside the kinase domain of Rad3p using a combination of genetic and biochemical methods. We find that they are required for full complementation of the checkpoint defects of *rad3Δ* cells and are also required for the catalytic activity of Rad3p. Using a genetic assay we have shown that the Rad3p N terminus contains at least two important sites, the leucine zipper and another novel site. Our results differ strikingly from previous studies of ATMp, which indicated that the isolated ATMp kinase domain is sufficient for complementation of A-T cell defects and for kinase activity (Baskaran *et al.*, 1997; Morgan *et al.*, 1997). Thus, there may be important differences in the regulation of the different classes of proteins within the PI3KR family.

MATERIALS AND METHODS

Strains

The strains used in this study are as follows: TE235 (*leu1–32 h*$^-$), TE236 (*leu1–32 ura4-D18 h*$^-$), and TE890 (*rad3::ura4*$^+$ *leu1–32 ura4-D18 h*$^-$). TE890 is a derivative of the *rad3::ura4*$^+$ strain created by Bentley *et al.* (1996). These strains were transformed with various *rad3* plasmids, which are listed in Table 1. To assay complementing activity of *rad3* alleles (see Figures 1 and 7), constructs were introduced into strain TE890. To assay dominant negative activity of *rad3* alleles (see Figures 3–6), constructs were introduced into strain TE235. To measure kinase activity (see Figures 2 and 7), constructs were introduced into strain TE890. For the coimmunoprecipitation experiments (see Figure 8), strain TE236 was transformed sequentially with two differentially marked plasmids, and *leu*$^+$*ura*$^+$ transformants were selected. In all cases, Rad3p expression from the transformed plasmids was controlled by the thiamine-repressible promoter *nmt1*$^+$ or a modified version of *nmt1*$^+$ termed the *nmt41* promoter (Basi *et al.*, 1993). Under the *nmt1*$^+$ promoter, proteins are induced ~80-fold when thiamine is removed from the media (Maundrell, 1990). The *nmt41* promoter has a mutated TATA box such that proteins are only induced ~12-fold (Basi *et al.*, 1993; Forsburg, 1993). Transformations were performed by electroporation (Prentice, 1992). Media and growth conditions were as previously described (Moreno *et al.*, 1991). Transformed strains were routinely maintained in Edinburgh minimal medium containing thiamine. To assay activity of Rad3 proteins, thiamine was washed out of the media to induce expression. It takes ~16 h to induce full expression of the *nmt* promoter by removing thiamine and ~20 h to repress it with the addition of thiamine (Maundrell, 1990). Fluorescence microscopy was performed using a Zeiss (Thornwood, NY) Axiophot microscope and a Photonic C1966 microscope image processor (Hamamatsu Photonic Systems, Bridgewater, NJ).

Description of Plasmids

The plasmids used in this study are listed in Table 1, and oligonucleotides used in their construction are listed in Table 2. The following is a brief description of the plasmids used in this study; detailed construction methods are available on request. Standard subcloning methods were used for all constructions. PCR reactions were carried out using *Pfu* polymerase according manufacturer's instructions (Stratagene, La Jolla, CA), and all PCR-amplified regions were sequenced. Protein expression from these plasmids was driven by either the *nmt1*$^+$ promoter (rep1) or the *nmt41* (rep41 or rep42) promoter (Basi *et al.*, 1993). The rep1 and rep41 plasmids are marked with the *S. cerevisiae LEU2* gene, which complements *S. pombe leu1–32* mutants, and the rep42 plasmids are marked with the *S. pombe ura4*$^+$ gene. Site positions are provided for restriction enzymes, using the A of the starting *rad3* ATG codon as position 1. rep1-*rad3*$^+$ was described previously (Bentley *et al.*, 1996) and con-

Table 1. DNA plasmids used in this paper

Plasmid number	Plasmid name	Source
pTE101	rep3x	
pTE119	rep41-HAtag	Bentley *et al.*, 1996
pTE120	rep42-myctag	Bentley *et al.*, 1996
pTE157	rep1-*rad3*$^+$	Bentley *et al.*, 1996
pTE165	pET3His-*rad3-Cterm*	This study
pTE446	rep1-*rad3-C725*	This study
pTE521	rep41-HA-*rad3*$^+$	This study
pTE541	rep1-HA-*rad3*$^+$	This study
pTE672	rep1-HA-*rad3-C725*	This study
pTE696	rep1-*rad3-N775*	This study
pTE697	rep1-HA-*rad3-N775*	This study
pTE698	rep1-*rad3-N690*	This study
pTE699	rep1-HA-*rad3-N690*	This study
pTE700	rep1-*rad3-N541*	This study
pTE701	rep1-HA-*rad3-N541*	This study
pTE706	rep1-*rad3-P*	This study
pTE707	rep1-HA-*rad3-P*	This study
pTE709	rep1-HA-*rad3-N775-P*	This study
pTE710	rep1-*rad3-N775-P*	This study
pTE715	rep1-HA-*rad3-LZ*	This study
pTE716	rep1-*rad3-LZ*	This study
pTE717	rep1-HA-*rad3-N775-LZ*	This study
pTE718	rep1-*rad3-N775-LZ*	This study
pTE743	rep1-*rad3-LZ-KD*	This study
pTE745	rep1-*rad3-LZ-P-KD*	This study
pTE746	rep41-*HA-rad3-LZ*	This study
pTE747	rep42-myc-*rad3-LZ*	This study
pTE748	rep42-myc-*rad3*$^+$	This study
pTE750	rep1-*rad3-P-KD*	This study
pTE783	rep1-HA-*rad3-KD*	This study
pTE784	rep1-HA-*rad3-C328*	This study
pTE785	rep1-HA-*rad3-C261*	This study
pTE786	rep1-HA-*rad3-C549*	This study
pTE787	rep1-HA-*rad3-C488*	This study
pTE791	rep1-*rad3-KD*	This study
pTE792	rep42-myc-*chk1*$^+$	This study

Table 2. Oligonucleotides used in this study

Oligo number	Oligo sequence
54	GGAATCCTGGCATATCATCAATTG
55	GCAGCTTGAATGGGCTTCCATAGT
154	TAATCATATGTATC
155	TCGAGATACATATGAT
196	TATGTTAAATGAAGGATCCG
197	GATCCTTCATTTAACA
198	TATGATGAATTCTCTTCA
199	TTTTGAAGAGAATTCATCA
200	CGGTGGAGCCAGCATTGCTCAAA
201	TTTAACAGAGCTCGAAATTATGTCGGG
210	GCATTAATCCCATGTACCCGTACGATGTTCCTGAC
217	TAAATAGTAGCTCGAG
225	CGCGTCATTAACTCGAG
226	TCGACACGAGTTAATGA
227	TCGACTCGAGCTACTATTTATGCA
234	TATGGCCTTAAGCCAAATGAT
235	ATCATTTGGCTTAAGGCCA
236	TATGAGTATGCCAAAATTGCTCACACT
237	GTGAGCAATTTTGGCATACTCA
263	GAAGAGGAAATTATGTCGACTTATTTTAGCAATGC
264	GAATTTATCTCTCCTAAGATGCGGGAGATTAAATCATCTGT
265	ACAGATGATTTAATCTCCCGCATCTTAGGAGAGATAAATTC

tains an *Nde*I site at position −2, and *Pst*I, *Sal*I, and *Bam*HI in the 3′ multicloning site. The following *rad3*$^+$ sites were used in cloning: *Bam*HI (4428), *Bsa*BI (1106), *Bsm*I (6391), *Dra*III (5534), *Ear*I (6188), *Eco*RV (5711), *Mlu*I (2062), *Nsi*I (2321), and *Xho*I (4987). A *Bst*EII site in the rep vector was also used. To generate rep42-myc-*chk1*$^+$, the *Nde*I–*Sac*I piece of rep1-*chk1*$^+$ (Carr *et al.*, 1995) was cloned into the rep42-myctag vector backbone (pTE120).

Tagged Wild-Type Rad3. To construct tagged versions of wild-type *rad3*$^+$ under control of the moderate strength *nmt* promoter, the *Nde*I–*Sal*I piece from rep1-*rad3*$^+$ (pTE157) was ligated into different vectors; for pTE521, rep41-HA-*rad3*$^+$, the rep41-HAtag vector was used (pTE119); for pTE748, the rep42-myctag vector (pTE120) was used. To construct pTE541, rep1-HA-*rad3*$^+$, in which hemagglutinin (HA)-tagged Rad3 expression is controlled by the wild-type *nmt1*$^+$ promoter, the HA tag from pTE119 was PCR amplified using oligos 210 and 55, such that the HA tag was flanked on the 5′ end with an *Ase*I site and at the 3′ end with an *Nde*I site. The resulting fragment was digested with *Ase*I and *Nde*I and cloned into the *Nde*I site of rep1-*rad3*$^+$ (pTE157).

C-terminal Fragments. pTE446, rep1-*C725*, is a deletion of *rad3*$^+$ sequences that encode amino acids 1–1660. Sequences between *Nde*I and *Xho*I were replaced with an *Nde*I–*Xho*I linker (oligos 154 and 155), which adds a starting methionine such that the sequence starts MYLE (Y = aa 1661). pTE672, rep1-*HA-C725*, contains the *Nde*I–*Sal*I fragment of pTE446 in the rep1-HA tag backbone of pTE541. pTE786, rep1-*HA-C549*, is a deletion of *rad3*$^+$ sequences that encode amino acids 1–1837. Sequences between *Nde*I and *Dra*III were replaced with an *Nde*I–*Dra*III linker (oligos 236 and 237), which adds a starting methionine such that the sequence after the HA tag starts MSMPK (S = aa 1838). pTE787, rep1-HA-*C488*, is a deletion of *rad3*$^+$ sequences that encode the first 1898 amino acids of Rad3p such that the sequence after the N-terminal HA tag starts MALSQ (A = aa 1899). It was constructed by using an *Nde*I–*Eco*RV linker (oligos 234 and 235) to replace *rad3*$^+$ sequences from *Nde*I to *Eco*RV by performing a three-way ligation with the linker, the *Eco*RV–*Sal*I fragment of pTE791, and the *Sal*I–*Nde*I fragment of the rep1HA tagging vector from pTE541. pTE784, rep1-*HA-C328*, is a deletion of *rad3*$^+$ sequences that encode amino acids 1–2058. Sequences between *Nde*I and *Ear*I were replaced with an *Nde*I–*Ear*I linker (oligos 198 and 199), which adds a starting methionine such that the sequence after the HA tag begins MMNSL (second M = aa 2059). pTE785, rep1-*HA-C261*, contains a deletion of *rad3*$^+$ sequences that encode amino acids 1–2125. Sequences between *Nde*I and *Bsm*I were replaced with an *Nde*I–*Bsm*I linker (oligos 196 and 197) such that the sequence after the HA tag starts MLNEGS (L = aa 2126, also GS is mutated from EC in wild-type Rad3p).

N-terminal Fragments. pTE696, rep1-*rad3-N775*, is a deletion of *rad3*$^+$ sequences that encode amino acids 776-2386. Sequences between *Nsi*I and *Sal*I of rep1-*rad3*$^+$ (pTE157) were replaced with an *Nsi*I-*Sal*I linker (oligos 217 and 227), which created an in-frame stop site after amino acid 775. pTE697, rep1-HA-*rad3-N775*, contains the same 3′ truncation as pTE696, in the rep1-HA backbone from pTE541. pTE698, rep1-*rad3-N690*, is a deletion of *rad3*$^+$ sequences 691-2386. Sequences between *Mlu*I and *Sal*I were replaced with a *Mlu*I–*Sal*I linker (oligos 225 and 226), which created a stop codon after amino acid 690. pTE699, rep1-HA-*rad3-N690*, is the same 3′ truncation as in pTE698, in the rep1-HA backbone from pTE541. pTE700, rep1-*rad3-N541*, is a deletion of *rad3*$^+$ sequences 542-2386. A stop codon and a *Sal*I site were inserted after amino acid 541 using PCR amplification with oligos 263 and 54, followed by subcloning. pTE701, rep1-HA-*N541*, contains the same 3′ truncation as pTE700 in the rep1-HA backbone from pTE541.

Leucine Zipper Deletion and Derivatives. pTE718, rep1-*rad3-N775-LZ*, contains the N775 C-terminal truncation with a 22 (Δ81–102)-amino-acid deletion of the leucine zipper. It was made by two-step PCR, using primers 264 and 55 and 265 and 54. Oligos 264 and 265 are primers that created the 22-amino-acid deletion of the leucine zipper. These products were then amplified again in the same tube with primers 54 and 55. A piece containing the deletion was then subcloned into pTE696, rep1-*rad3-N775*. pTE717, rep1-HA-*rad3-N775-LZ*, contains the same 22-amino-acid deletion (Δ81–102) as pTE718 but in the rep1-HA backbone of pTE541. pTE716, rep1-*rad3-LZ*, contains the leucine zipper deletion (pTE718) in full-length *rad3* in the rep1 vector. pTE715, rep1-HA-*rad3-LZ*, contains the leucine zipper deletion (from pTE718) in full-length *rad3* in the rep1 HA tagging vector. pTE746, rep41-HA-*rad3-LZ*, contains the leucine zipper deletion (from pTE718) in full-length *rad3* in the rep41 HA tagging vector. pTE747, rep42-myc-*rad3-LZ*, contains the full-length *rad3* sequence with the leucine zipper deletion in the rep42 myc tagging vector (pTE120).

P-Site Deletion and Derivatives. pTE706, rep1-*rad3-P*, which harbors a nine-amino-acid deletion (Δ551–559) marked by a silent *Sac*I site (1642), was created in a series of steps, which included whole-plasmid PCR mutagenesis with primers 200 and 201 using the protocol described in Stratagene's QuikChange site-directed mutagenesis kit instruction manual. pTE707, rep1-HA-*rad3-P*, consists of the *Nde*I–*Sal*I piece of pTE706 in the rep1-HA backbone of pTE541. pTE710, rep1-*rad3-N775-P*, which contains the P-site deletion in the N775 truncation construct, was created by ligating the *Nde*I–*Nsi*I insert piece from pTE707 into the similarly cut backbone of pTE696.

Kinase-dead Alleles of rad3. pTE791, rep1-*rad3-KD*, which encodes a kinase-dead form of Rad3p under the control of the *nmt1*$^+$ promoter, was constructed by replacing the *Bam*HI–*Pst*I fragment of pTE157 (rep1-*rad3*) with the equivalent piece from a vector containing the *rad3.a* allele (Bentley *et al.*, 1996), which contains an AT→CG mutation at 6689–6690 and thus encodes a kinase-dead (D2230A) form of Rad3p. pTE783, rep1-HA-*rad3-KD*, which encodes an N-terminally HA-tagged kinase-dead form of Rad3p, was constructed by cloning the *Bam*HI–*Bam*HI piece of pTE791 into the similarly cut backbone of pTE541, rep1-HA-*rad3*. pTE743, rep1-*rad3-LZ-KD*, which contains the leucine zipper deletion in the context of the full-length *rad3* kinase-dead D2230A allele, was constructed by subcloning the *Nde*I–*Nsi*I insert piece of pTE715 into the similarly cut backbone of pTE791. pTE750 contains the P-site deletion in full-length *rad3* dominant negative D2230A allele and was made by subcloning the *Nde*I–*Nsi*I insert from pTE710 into the similarly cut backbone of pTE791. pTE745, rep1-*rad3-LZ-P-KD*, contains the leucine zipper deletion, the P-site deletion, and the kinase-dead D2230A mutation in the context of full-length *rad3*. This construct was made by using a three-way ligation to combine the following appropriate pieces: *Bst*EII–*Nsi*I of pTE791, *Bst*EII–*Bsm*I of pTE718, and *Bsm*I–*Nsi*I of pTE707.

Hydroxyurea Sensitivity Assays

Liquid Assays. In these assays, *rad3* alleles were overexpressed from the full-strength *nmt1*$^+$ promoter. Overnight cultures of cells were grown in the presence of thiamine to repress transcription of *rad3* alleles. To induce transcription, these cultures were diluted into media lacking thiamine and grown for 20 h to an OD_{595} between 0.1 and 0.2. At this time, 10 mM hydroxyurea (HU) was added, and the cultures were incubated at 29°C with shaking. Samples were taken every 2 h, plated in duplicate on solid media containing thiamine, and incubated at 29°C for 3–5 d. Two time courses were done for each experiment, and the data points on the graphs represent the average of the results of these time courses.

Plate Assays. As in the liquid assays, *rad3* alleles were expressed from the full-strength *nmt1*$^+$ promoter. Strains were initially streaked onto plates of media containing thiamine. These plates were incubated at 29°C for 2–3 d and replica plated onto media lacking thiamine to induce expression of the *rad3* alleles. After 1–2 d of growth at 29°C, these plates were replica plated again, to plates lacking thiamine and containing 10 mM HU. Pictures of the plates were taken after 3 d of growth on HU at 29°C.

UV Sensitivity Assays

For UV sensitivity assays, Rad3p expression was induced as described above for the HU liquid assays. After 20 h of induction, samples were plated on solid media containing thiamine. The plates were dried at room temperature for 1–2 h, irradiated in a UV Stratalinker 2400 with doses of UV irradiation from 0 to 200 J/m^2, and then incubated at 29°C for 3–5 d. It takes at least 20 h to repress the *nmt* promoter under these conditions so at the time of irradiation Rad3 proteins are still expressed at high levels; however, for most of growth afterward at 29°C, the thiamine in the plates represses expression of Rad3p from the *nmt* promoter. The numbers of colonies on duplicate plates for each UV dose were averaged, and viability for each dose was calculated using the number of colonies on the mock-irradiated (0 J/m^2) plate as a viability of 1.0. The graphs presented in this paper are each the average of results from two dosage series.

Fluorescence-activated Cell-sorting (FACS) Analysis

Samples for FACS analysis were prepared as described (Sazer and Sherwood, 1990), except that ethanol-fixed cells were stored at −20°C before processing for propidium iodide staining. FACS analysis was performed with a FACSCalibur cytometer and Cell Quest version 3.1f software (Becton Dickinson, San Jose, CA). Ten thousand events were counted for each sample. FACS data were gated to plot cells with an FL2 area of 68–520 and smoothed by a factor of 5.

Polyclonal Antibodies to Rad3p

To produce Rad3p in bacteria, the C-terminal region of Rad3p (the *Bam*HI–*Bam*HI piece of rep1-*rad3*$^+$ [pTE157]) was cloned in-frame into the *Bam*HI site of the pET3-His *Escherichia coli* histidine-tagging vector (Chen and Hai, 1994) and transformed into BL21 cells. Expression was induced by adding 0.5 mM isopropyl-1-thio-α-D-galactopyranoside to an exponentially growing culture for 3 h at 25°C. The bacterial pellet was resuspended in IMAC5 (20 mM Tris-Cl, pH 8.0, and 0.5 M NaCl, with 5 mM imidazole) containing 1 mg/ml lysozyme and 0.1% NP-40, sonicated, and centrifuged. The insoluble pellet was solubilized with IMAC5 containing 6 M GuHCl and bound by batch method to Talon Ni^{2+} agarose (Clontech, Palo Alto, CA) and washed with IMAC5, IMAC10, and IMAC20 all containing 6 M GuHCl, followed by H_2O. Rad3p protein was eluted from the Ni^{2+} beads by boiling into 2% SDS and 10 mM EDTA, dialyzed against 1% SDS, 0.025 M Tris, 0.192 M glycine, and 10 mM EDTA, and injected into rabbits (Cocalico Biologicals. Reamstown, PA).

Protein Electrophoresis and Western Blotting

All full-length Rad3p samples were resolved on 5% gels for ~16 h at 100 V (as described by Scully *et al.*, 1997). Smaller proteins were resolved on 10 or 12% gels. A BenchMark prestained protein ladder (Life Technologies, Gaithersburg, MD) was used for molecular weight markers. Transfer to Immobilon-P (Millipore, Bedford, MA) was performed in a semidry apparatus (Owl Scientific, Woburn, MA) using the transfer buffer described by Scully *et al.* (1997). Blots were then dried after brief immersion in methanol. Once dry, the blots were briefly resoaked in methanol, washed with H_2O, and blocked with 1× Tris-buffered saline (TBS) containing 1% dry milk and 1% BSA. Primary antibody was added for one h in 1× TBS and 0.05% Tween 20 (TBST). For HA blots, monoclonal anti-HA anti-

body, clone 12CA5, was diluted 1:5000 (a generous gift from Ed Harlow, Harvard Medical School, Boston, MA); for myc blots, the 9E10 monoclonal antibody was diluted 1:200 (M5546; Sigma, St. Louis, MO). The Rad3p HM126 polyclonal antibody serum was diluted 1:5000. After washing, secondary antibody (HRP-conjugated anti-mouse or anti-rabbit; Amersham, Arlington Heights, IL) was added at a 1:5000 dilution in 1× TBST. After washing with 1× TBST, blots were developed using ECL (Amersham).

Immunoprecipation Assays

Yeast protein extracts were prepared from early log phase cultures, grown in the absence of thiamine for 20–24 h, to induce protein expression from the attenuated *nmt* promoter (rep41/rep42) for the coimmunoprecipitation assays, or from *nmt1*$^+$ for the kinase assay experiments. Immunoprecipitations were performed as described by Bentley *et al.* (1996) with the following modifications. Cells (4×10^8) were lysed with glass beads into immunoprecipitation buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% NP-40, 50 mM NaF, 60 mM β-glycerophosphate, 1 mM $NaVO_4$, 2 mM PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin) in a BIO 101 (La Jolla, CA) Fast-Prep. Primary antibody (anti-myc clone 9E10, Sigma M5546, or anti-HA 12CA5, a generous gift from Ed Harlow) was preincubated with protein G (anti-myc)- or protein A (anti-HA)-Sepharose beads in lysis buffer at 4°C for 1 h on a rotating wheel and then washed in lysis buffer. Beads were then divided, combined with extract, and incubated on a rotating wheel for 1 h at 4°C. After washing the beads five times with lysis buffer, the beads were split, and 2× SDS sample buffer was added. Samples were then boiled and loaded onto SDS-PAGE gels, as described above in Protein Electrophoresis and Western Blotting. To check expression of various proteins in total cell extracts, cells were lysed with acid-washed glass beads directly into 2× SDS sample buffer in a BIO 101 Fast-Prep.

Kinase Assays

For these assays, *rad3* alleles were expressed from the full-strength *nmt1*$^+$ promoter. Yeast protein extracts for immunoprecipitations were prepared as described above in Immunoprecipitation Assays. After washing the immunoprecipitates, they were split in half. Half was used for kinase assays, whereas the other half was Western blotted to visualize Rad3p as described above. The half of the precipitate to be used for the kinase assay was washed once in kinase buffer without substrate or ATP (25 mM HEPES, pH 7.7, 50 mM KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1% NP-40, 2% glycerol, 1 mM DTT, 1 mM $NaVO_4$). Ten microliters of kinase buffer containing substrate and ATP (kinase buffer containing 10 mM ATP, 5–10 μCi of [γ^{32}P]ATP, 1 μg of PHAS-1) was added to each immunoprecipitate, and the reactions were incubated for 15 min at 30°C at which point 10 μl 2× SDS sample buffer containing 10 mM EDTA was added to each to stop the reactions. PHAS-1 protein was a generous gift from Merl Hoekstra (Signal Pharmaceuticals, San Diego, CA). Each sample was heated at 95°C for 2–5 min and then resolved by SDS-PAGE on a 12 or 15% gel. Gels were Coomassie blue stained, fixed, and dried under vacuum for 2 h with low levels of heat (50–60°C). Bands were visualized by autoradiography of the dried gels for 3–12 h at −70°C. Assays for wortmannin sensitivity were done as described with the following exceptions. After the immunoprecipitates were split, they were washed twice in wash buffer (25 mM HEPES, pH 7.7, 50 mM KCl, 10 mM $MgCl_2$). Wortmannin diluted in the above wash buffer was then added in a volume of 100 μl in various concentrations, and the precipitates were incubated at room temperature for 20 min. The wortmannin was inactivated with the addition of kinase buffer without substrate or ATP, and the precipitates were then washed once with kinase buffer without substrate or ATP.

RESULTS

The Rad3p Kinase Domain Is Not Sufficient for Checkpoint Function

Rad3p is a large, 2386-amino-acid protein. Thus far, the only region of Rad3p shown to be important for function is the C-terminal PI3-like kinase domain that makes up <15% of the Rad3 protein (Bentley *et al.*, 1996). To determine whether sites outside the kinase domain are required for Rad3p activity, we examined the biological activity of a series of C-terminal fragments of Rad3p shown in Figure 1A. All but one of these HA epitope-tagged truncations contains the full kinase domain (Bentley *et al.* 1996). Of these constructs, *rad3-C328* encodes a protein that is most similar to the kinase domain fragment used in studies of ATMp (Morgan *et al.*, 1997). The ability of these constructs to rescue a *rad3*Δ strain was determined. Because *rad3*Δ cells are checkpoint defective, they are exquisitely sensitive to the DNA replication inhibitor HU and to DNA damage induced by UV radiation.

As shown in Figure 1B, *rad3-C328* exhibits very little complementing activity in the HU assay, because cells expressing this construct were almost as sensitive to HU as cells transformed with empty vector. Although the larger fragments *rad3-C549* and *rad3-C725* more significantly improved the viability of the *rad3*Δ strain after HU treatment (Figure 1B), the transformants were still 10 times more HU sensitive than cells transformed with full-length *rad3*$^+$. Two other fragments, *rad3-C488* and *rad3-C261*, also exhibited complementing activity significantly below that of full-length *rad3*$^+$ (our unpublished results). Radiation sensitivity of transformants was also analyzed as shown in Figure 1C. None of the truncated alleles showed any complementing activity in this assay (Figure 1C and our unpublished results), although they all directed high levels of protein expression (Figure 1D). We conclude that the isolated kinase domain cannot perform wild-type Rad3p function. This may be because N-terminal sequences are crucial for biological activity. Alternatively, we may have eliminated sequences necessary for correct cellular localization of the protein. In contrast, a 390-amino-acid kinase domain fragment of ATMp, equivalent to the last 355 amino acids of Rad3p, is sufficient for at least some activities (Baskaran *et al.*, 1997; Morgan *et al.*, 1997).

The C-terminal fragments cannot fully rescue the sensitivities of the *rad3*Δ mutants in either the HU or UV assays. However, several of the mutant proteins are able to partially restore resistance to HU but not to UV. A similar phenomenon is described below for the Rad3-KD protein (see Figure 3). It is possible that the observed differences reflect quantitative or qualitative differences in the assays. For example, the cells are arrested in S phase in the HU assay, whereas most of the cells are in G2 when they are irradiated with UV. An interesting alternative is that the mutant proteins exhibit varying abilities to complement (or disrupt) the DNA replication and DNA damage responses because these two responses have different requirements for certain Rad3p functions.

Sequences Outside the Kinase Domain of Rad3p Are Required for Kinase Activity

The isolated kinase domain may not complement *rad3*Δ cells, because it is unable to interact with cofactors or sub-

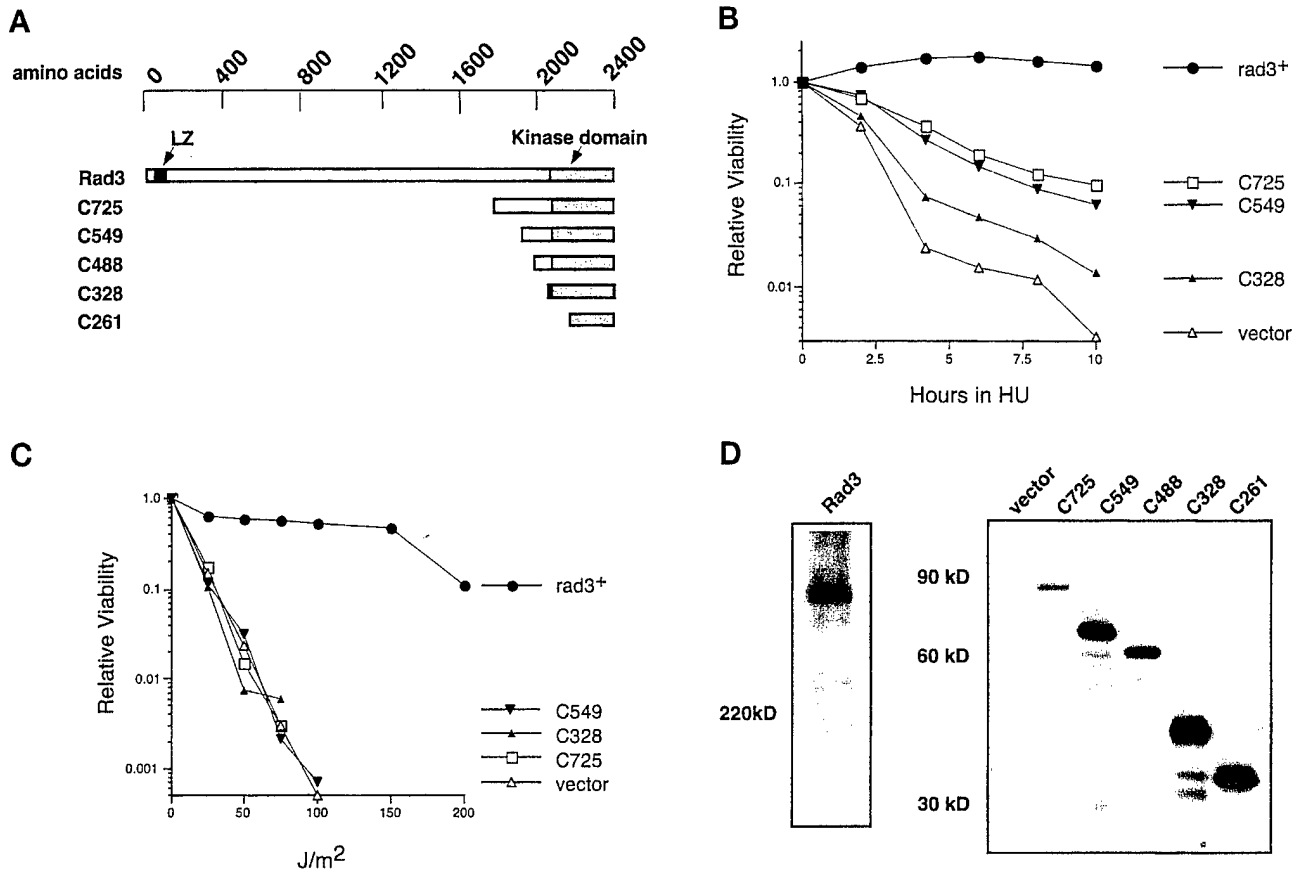

Figure 1. The isolated Rad3p kinase domain does not complement *rad3*Δ cells. (A) Rad3p kinase domain fragments are diagrammed approximately to scale. The positions of the kinase domain (shaded) and the leucine zipper motif (black) are indicated. (B) Relative viability of *rad3*Δ cells (TE890) expressing HA-tagged mutant Rad3 proteins, after indicated periods of incubation in 10 mM HU. SE analysis of these curves indicates that their positions are significant. The plasmids used are rep1-HA-*rad3*$^+$(pTE541), rep1-HA-*rad3-C725* (pTE672), rep1-HA-*rad3-C549* (pTE786), rep1-HA-*rad3-C328* (pTE784), and rep3x (pTE101). These proteins are overexpressed because their expression is controlled by the full-strength *nmt1*$^+$ promoter. Note that rep1-HA-*rad3*$^+$ fully complements the *rad3*Δ strain, exhibiting wild-type levels of viability in both HU and UV assays (our unpublished results). (C) Relative viability of the same strains after irradiation with the indicated doses of UV. The results shown are the average of two independent experiments. No colonies were recovered at doses at which no points are shown. (D) Expression of full-length Rad3p and Rad3p C-terminal fragments. Left panel, Western blot of total extracts prepared from *rad3::ura4*$^+$ *leu1–32 h*$^-$ cells (TE890) and cells transformed with rep1-HA-*rad3*$^+$ (pTE541). Right panel, Western blot of total extracts prepared from the transformants used in B and C, as well as *rad3*Δ cells (TE890) transformed with rep1-HA-*rad3-C261* (pTE785) and rep1-HA-*rad3-C488* (pTE787). Both blots were probed with monoclonal antibodies specific for the HA epitope tag (12CA5). Note that direct comparison of levels of expression of full-length to truncations is not feasible, because the different proteins are resolved on different percentage gels and are likely to have different transfer efficiencies. However, we estimate that the truncations are three- to fivefold more abundant than the full-length protein.

strates. Alternatively, sequences outside the kinase domain may be directly required for Rad3p catalytic activity. To investigate this possibility, we examined the catalytic activity of the Rad3p C-terminal fragments examined above. Rad3p kinase activity was measured by in vitro phosphorylation of an exogenous substrate, the eukaryotic initiation factor-4E (eIF-4E) binding protein, PHAS-1, a human translational regulatory protein also phosphorylated by the Rad3p homologues ATMp and ATRp (Banin *et al.*, 1998; Canman *et al.*, 1998; Sarkaria *et al.*, 1998). High levels of PHAS-1 phosphorylation were readily observed when full-length Rad3p was incubated with the substrate (Figure 2, A, lane 2, and B, lane 2) so that a qualitative assessment of Rad3p activity could be easily made visually. As previously reported (Bentley *et al.*, 1996), autophosphorylation of Rad3p was also observed under these conditions (our unpublished results); however, because the levels of autophosphorylation were much lower than the levels of PHAS-1 phosphorylation in this assay, PHAS-1 phosphorylation was used to measure Rad3p activity. Importantly, the kinase activity observed is likely to be due specifically to the Rad3 protein and not another coimmunoprecipitating protein, because phosphorylation of PHAS-1 was not observed in immunoprecipitates of extracts from cells expressing *rad3-KD*, which encodes a protein carrying the mutation, D2230A, predicted to abolish kinase activity (Bentley *et al.*, 1996) (Figure 2, A and B). No phosphorylation of PHAS-1 was observed in immunoprecipitates from any of the strains transformed

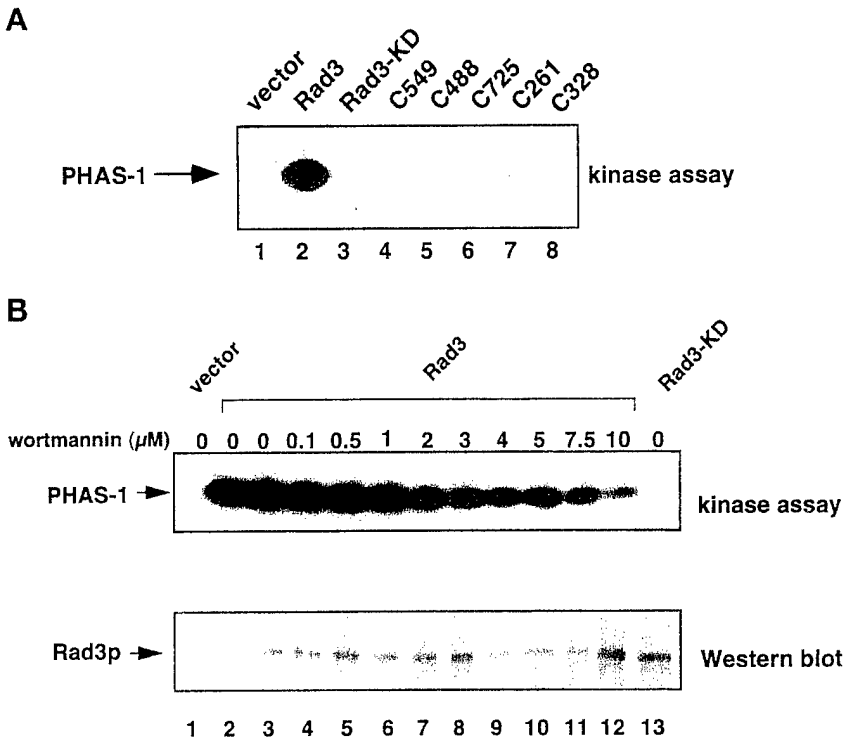

Figure 2. Characterization of Rad3p kinase activity. (A) The isolated kinase domain of Rad3p is not catalytically active. HA-tagged Rad3 proteins were immunoprecipitated from *rad3Δ* (TE890) transformed with rep3x (pTE101), rep1-HA-*rad3*$^+$ (pTE541), rep1-HA-*rad3-C549* (pTE786), rep1-HA-*rad3-C488* (pTE787), rep1-HA-*rad3-C725* (pTE672), rep1-HA-*rad3-C261* (pTE785), rep1-HA-*rad3-C328* (pTE784), or rep1-HA-*rad3-KD* (pTE783). These proteins are overexpressed because their expression is under the control of the *nmt1*$^+$ promoter. Immunoprecipitates were divided in half and either assayed for their ability to phosphorylate PHAS-1 (top panel) or Western-blotted and probed with anti-HA antibody (our unpublished data). (B) Rad3p is relatively insensitive to wortmannin. HA-tagged Rad3 proteins were immunoprecipitated from *rad3Δ* (TE890) transformed with rep3x (pTE101), rep1-HA-*rad3*$^+$ (pTE541), or rep1-HA-*rad3-KD* (pTE783). HA immunoprecipitates from the indicated strains were assayed for their ability to phosphorylate PHAS-1 in the presence of increasing concentrations of wortmannin.

with C-terminal truncations of *rad3*$^+$ (Figure 2A, lanes 4–8), although Western blot analysis showed that the fragments were expressed and precipitated at levels equal to or greater than wild-type Rad3p (Figure 1D and our unpublished results). Because several of the C-terminal fragments of Rad3p are able to slightly improve the viability of the *rad3Δ* strain in the presence of HU, we have also examined the kinase activity of the Rad3-C725p, Rad3-C488p, and Rad3-C328p C-terminal fragments in the presence of HU. Cells overexpressing these fragments were treated with 10 mM HU for 3 and 6 h. Rad3p C-terminal fragments immunoprecipitated from these extracts did not exhibit any activity toward PHAS-1 (our unpublished results). We conclude that sequences N-terminal to the kinase domain are required for Rad3p catalytic activity. In contrast, the isolated kinase domain of ATMp (a 390-amino-acid C-terminal fragment) has been shown to phosphorylate c-Abl (Baskaran *et al.*, 1997).

Rad3p Is Relatively Resistant to the PI3-Kinase Inhibitor Wortmannin

Although *rad3Δ* cells share some phenotypes with A-T cells, suggesting that there may be some functional overlap between Rad3p and ATMp, the primary amino acid sequence of Rad3p is more similar to human ATRp than to ATMp. In contrast to ATMp, regions outside of the kinase domain are absolutely required for Rad3p kinase activity, suggesting that the conformation of the catalytic sites of these proteins may be different. The drug wortmannin, which irreversibly inhibits PI3-kinases by modifying an invariant lysine residue in the catalytic site, has been used to further characterize the relationships among the PI3KR kinases. ATMp is 10 times more sensitive to wortmannin (IC_{50}, 150 nM) than ATRp (IC_{50}, 1.8 μM) (Banin *et al.*, 1998; Sarkaria *et al.*, 1998). Using the kinase assay described above, the sensitivity of Rad3p to wortmannin was examined. As shown in Figure 2B, Rad3p retained close to wild-type levels of activity in the presence of 1 μM wortmannin (lane 6). Approximately 50% inhibition of Rad3p activity occurs when concentrations of 2–4 μM of wortmannin are added. In the presence of a 10 μM concentration of wortmannin, Rad3p activity is decreased ~10-fold, indicating that the wortmannin is active. Although the kinase assay is a qualitative assay in which activity is assessed visually, it allows us to distinguish between inhibition in the micromolar and nanomolar range. In contrast to the relatively high resistance of Rad3p to wortmannin, ATM and DNA-PK protein kinase activity was completely abolished by a 1 μM concentration of this lot of wortmannin (Rathbun, unpublished observations). Thus, the conformation of the catalytic site of Rad3p likely resembles that of ATRp more than that of ATMp.

Overexpression of the N-terminal 775 Amino Acids of Rad3p Disrupts the Checkpoint Response of Wild-Type Cells

The above studies indicate that sequences N-terminal to the kinase domain of Rad3p are required for its catalytic activity. However, they do not address the function of the N terminus in interactions with regulators or substrates. To explore this possible role for the N-terminal sequences, we examined the ability of fragments of Rad3p to function as dominant negative alleles, that is, to disrupt checkpoint control of wild-type cells when overexpressed. It has been

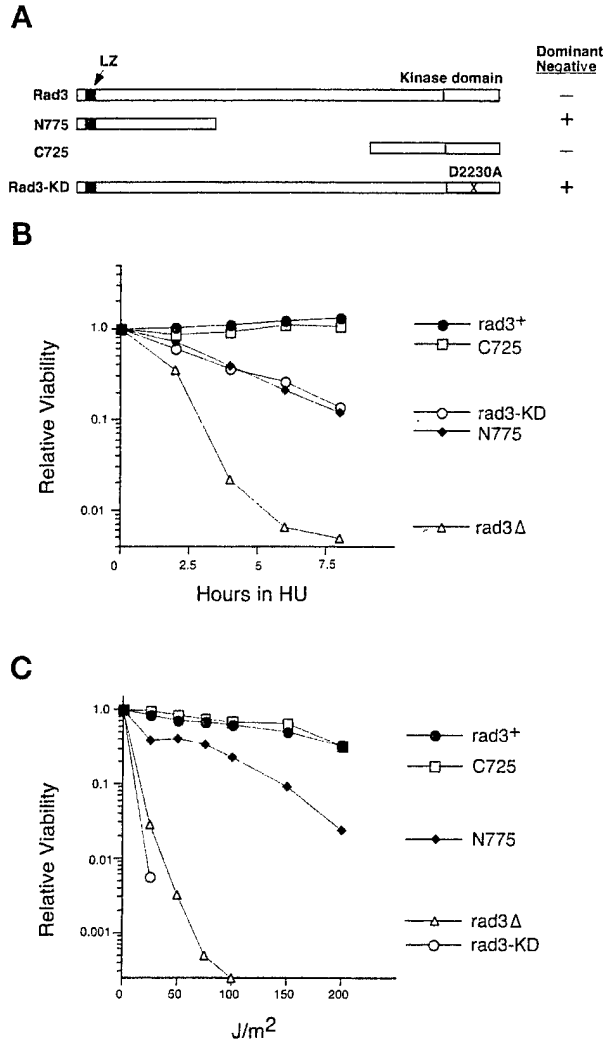

Figure 3. Overexpression of an N-terminal fragment of Rad3p disrupts the response of wild-type cells to HU and UV. (A) Diagram of mutant Rad3 proteins, drawn approximately to scale. See Figure 1 for explanation of symbols. Dominant negative activity of each mutant protein is indicated. (B) Relative viability after treatment with 10 mM HU of wild-type cells (TE235) expressing *rad3*$^+$ (pTE157), *rad3-C725* (pTE446), *rad3-KD* (pTE791), *rad3-N775* (pTE696), and rep3x (pTE101). (C) Relative viability of the same strains with indicated doses of UV irradiation. No colonies were recovered at doses at which no points are shown. The results shown are the average of two independent experiments.

shown previously that *rad3-KD* acts as a dominant negative when overexpressed in wild-type cells, presumably because the catalytically incompetent protein sequesters key regulators or substrates into nonfunctional complexes (Bentley *et al.*, 1996). To define the regions of Rad3p that compete with the wild-type protein, *rad3*$^+$ truncations were overexpressed in wild-type cells, and their ability to disrupt HU and UV resistance was determined (Figure 3). Overexpression of *rad3-N775*, which encodes the N-terminal 775 amino acids of Rad3p, greatly increased the sensitivity of wild-type cells to both HU and UV (Figure 3, B and C). These data parallel results with the ATM protein in which an N-terminal fragment can disrupt checkpoint function of human cells (Morgan *et al.*, 1997). Expression of a C-terminal truncation that includes the full kinase domain, *rad3-C725*, did not affect the HU or UV resistance of wild-type cells.

Although overexpression of *rad3-N775* significantly disrupted the UV and HU responses, it behaves differently from the dominant negative *rad3-KD* allele in a few notable ways. For example, cells overexpressing *rad3-N775* are much less UV sensitive than cells overexpressing *rad3-KD*. In addition, cells overexpressing *rad3-N775* are more varied in cell length (Figure 4B), even in the absence of HU (our unpublished results). As previously reported, *rad3-KD* overexpression is lethal to cells over many generations (Bentley *et al.*, 1996); in contrast, overexpression of *rad3-N775* does not grossly affect cell viability.

To determine whether the HU sensitivity caused by overexpression of these constructs is due to disruption of cell cycle checkpoints, we examined mitosis and DNA replication in the presence of HU in these strains and in *rad3*Δ and wild-type cells. As shown in Figure 4A, *rad3*Δ cells and cells overexpressing *rad3-KD* and *rad3-N775* undergo abnormal mitoses ("cuts") in which the nucleus is cleaved by a septum, or anucleate cells are generated; this is typically observed when cells enter mitosis with less than fully replicated DNA. The *rad3-KD* and *rad3-N775* cultures continue to accumulate cuts for the next 6 h with identical kinetics, although ~20% fewer cuts are observed in cells overexpressing the mutants compared with *rad3*Δ cells. In contrast, no cuts are observed in cells transformed with *rad3*$^+$ or *rad3-C725*. Photographs of cells treated with HU for 10 h are shown in Figure 4B.

To determine whether the abnormal mitoses are due to cells entering mitosis with unreplicated DNA, we also examined DNA content by FACS analysis during a 10-h HU exposure. As shown in Figure 4C, before HU treatment, most of the cells have a 2C DNA content, as is characteristic of fission yeast cultures, because G2 constitutes 80% of the fission yeast cell cycle. After 2 h in HU, a 1C peak is evident, and by 4 and 6 h the majority of the cells in all the cultures show a 1C DNA content indicating that they are blocked from completing S-phase by HU. Notably, by 4 and 6 h significant numbers of cuts are observed in *rad3*Δ, *rad3-KD*, and *rad3-N775* cultures, although FACS analysis indicates that they have not completed DNA replication by this time. From these results we conclude that mutant overexpression, like the loss of *rad3*, causes defects in the checkpoint that makes mitosis dependent on completion of DNA replication. The similarity in the kinetics of the appearance of cuts in all the affected cultures suggests that defects in checkpoint control caused by mutant overexpression are qualitatively similar to the well-characterized defect of *rad3*Δ cells, although they are quantitatively less severe. As previously noted (Figure 3B), *rad3*Δ cells are significantly more sensitive to HU. Thus, although the overexpressed mutant proteins significantly disrupt the mitotic checkpoint, other Rad3p functions that are missing in the deletion, such as regulation of S-phase (Lindsay *et al.*, 1998) and recovery (Enoch *et al.*, 1992), may still be intact.

The FACS analysis also reveals that wild-type cells and *rad3*Δ cells begin to synthesize DNA after 6 h of HU

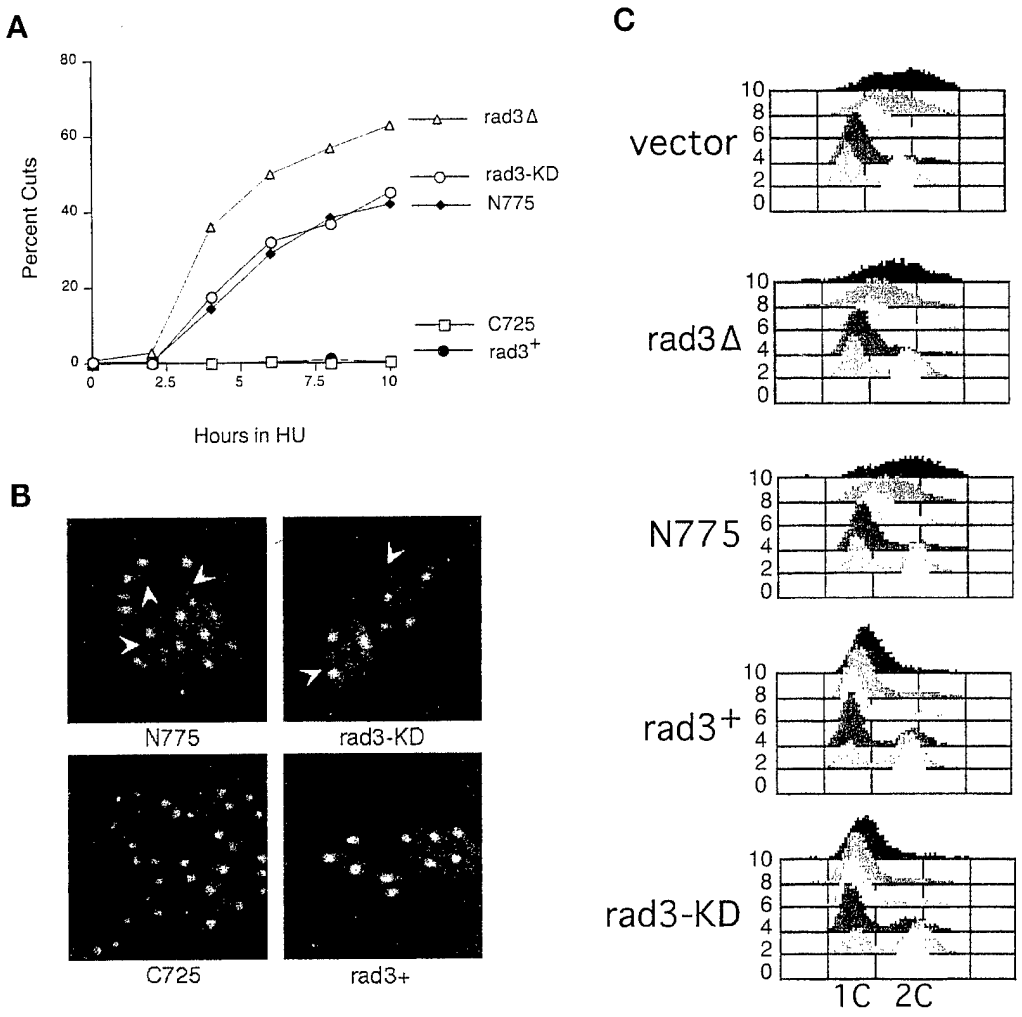

Figure 4. Rad3p dominant negative alleles disrupt the checkpoint coupling mitosis to completion of DNA replication. (A) Percent aberrant mitoses after treatment with 10 mM HU of wild-type cells (TE235) expressing *rad3*$^+$ (pTE157), *rad3-C725* (pTE446), *rad3-KD* (pTE791), *rad3-N775* (pTE696), and rep3x (pTE101). The results shown are the average of two independent experiments. (B) Photomicrographs of cells cultured in 10 mM HU for 10 h and then fixed and stained with the DNA-specific dye DAPI. Cells were wild-type (TE235) expressing *rad3-N775*, *rad3-KD*, *rad3-C725*, and *rad3*$^+$. Aberrant mitoses are indicated with arrows. (C) FACS analysis of cells incubated with HU for indicated times (at left). Wild-type cells expressing rep3x control (vector), *rad3-N775* (N775), *rad3*$^+$, and *rad3-KD* are shown, as well as *rad3*Δ cells expressing the rep3x vector (*rad3*Δ). Note that overexpression of *rad3*$^+$ or *rad3-KD* inhibits DNA replication after exposure to HU. See text for more details.

exposure, and synthesis continues inefficiently for the next 4 h. This slow escape from HU arrest has previously been described by others (Sazer and Nurse, 1994). In remarkable contrast, DNA synthesis in the presence of HU is significantly inhibited by overexpression of *rad3*$^+$ or *rad3-KD*, because even by 10 h the majority of the cells in the culture still have a 1C DNA content. In contrast, overexpression of *rad3-N775* has no effect on this aspect of the HU response. These results suggest that overexpression of *rad3*$^+$ or *rad3-KD* inhibits DNA replication under some circumstances. Additional studies of the effects of *rad3*$^+$ overexpression on DNA replication are under way and will be presented elsewhere (Chapman and Enoch, unpublished data).

The Leucine Zipper Is Required but Not Sufficient for Dominant Negative Activity of rad3-N775

Expression of *rad3-N775* may disrupt the checkpoint response by sequestering Rad3p-interacting proteins into nonproductive complexes. Such a model predicts that the dominant negative activity of *rad3-N775* will be abolished by mutations that eliminate binding sites for Rad3p-interacting proteins. Thus we hypothesized that by examining the dominant negative activity of *rad3-N775* mutants, it should be possible to identify regions of Rad3p that mediate critical interactions with other cellular proteins.

Like other PI3KR kinases, the N terminus of Rad3p contains a putative leucine zipper. Because leucine zipper

motifs frequently mediate protein–protein interactions (Phizicky and Fields, 1995), this site in Rad3p was a logical candidate for a domain required for dominant negative activity. To examine the role of this putative leucine zipper, 22 amino acids, from the first leucine to the last leucine, were deleted from the *rad3-N775* construct. The dominant negative activity of the resulting allele (*rad3-N775-LZ*) was tested. As shown in Figure 5A, wild-type cells transformed with *rad3-N775-LZ* grew almost normally on plates containing HU, in contrast to cells transformed with *rad3-N775*. For comparison, *rad3Δ* cells and wild-type cells are also shown. We conclude that the leucine zipper is likely to interact with factors that are limiting for the checkpoint response.

When a series of smaller N-terminal truncations was examined, evidence for another site involved in the dominant negative activity was found. The deletion series used for this analysis is shown in Figure 5C. We found that a fragment encoding the N-terminal 690 amino acids of Rad3p (*rad3-N690*) retained dominant negative activity; however, truncation of an additional 150 amino acids (*rad3-N541*) eliminated this activity. Because *rad3-N541* contains the leucine zipper motif, we conclude that overexpression of this motif is not sufficient for dominant negative activity. This result also suggested that another important protein-protein interaction domain might be located in the region between amino acids 541 and 690. Interestingly, this region contains the motif QSLLLDGFF, at amino acids 551–559, which closely resembles the consensus (QXXI/LXXFF) for a domain that mediates an interaction with proliferating cell nuclear antigen (PCNA), the processivity factor for DNA polymerase (Warbrick *et al.*, 1995; Montecucco *et al.*, 1998). To determine whether this site is required for the dominant activity of *rad3-N775*, a mutant deleted for these nine amino acids of this putative PCNA binding site (*rad3-N775-P*) was constructed and tested for its ability to render wild-type cells HU sensitive. As shown in Figure 5B, deletion of this site which we call the "P-site" abolishes the dominant negative activity of *rad3-N775*. We conclude that both the P-site and the leucine zipper may interact with proteins required for the checkpoint response. However, it is possible that mutation of these sites disrupts dominant negative activity for other reasons; for example, the deletions may affect local structure of the protein. To definitively show that these sites are indeed protein-protein interaction sites, a protein that shows a leucine zipper (LZ) or P-site-dependent interaction with Rad3p must be identified.

We also examined the dominant-negative activity of HA-tagged versions of these N-terminal alleles. Again, overexpression of HA-*rad3-N775* disrupted the checkpoint response of wild-type cells, however, not as dramatically as the untagged allele, suggesting that the presence of the HA tag may interfere with some Rad3p interactions. The dominant negative activity of the HA-tagged allele could also be abolished by deleting the leucine zipper or the P-site, confirming the significance of these sites. Western blots established that the HA-tagged versions of all these alleles were expressed at relatively equal levels, establishing that the deletions do not affect protein stability (Figure 5D). None of these alleles (tagged or untagged) has gross effects on cell viability when cells are grown in the absence of HU.

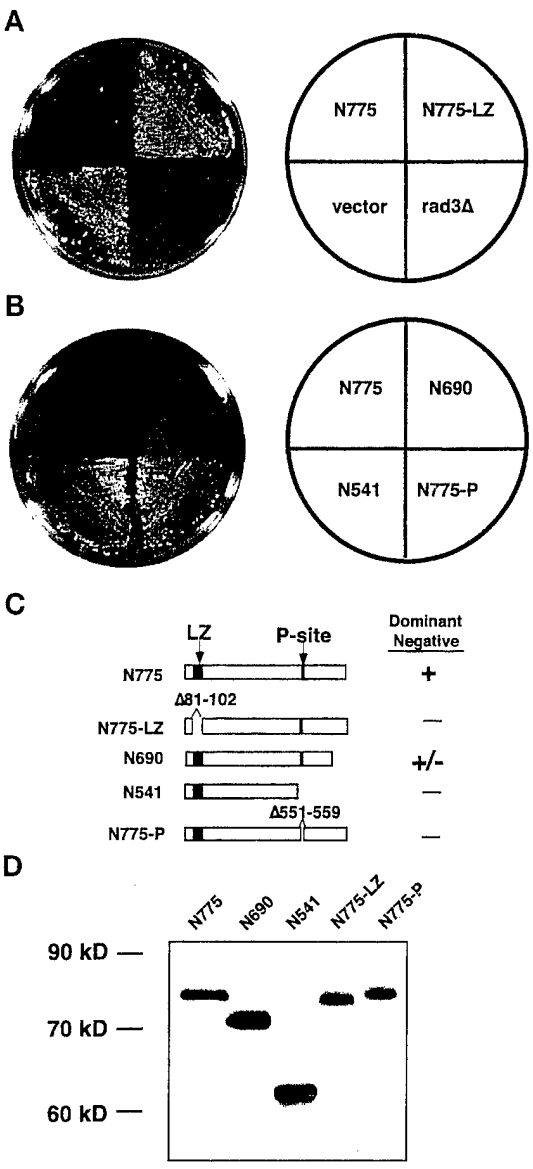

Figure 5. Identification of regions necessary for dominant negative activity of Rad3-N775p. (A) Leucine zipper motif is required for dominant negative activity of Rad3-N775p. Wild-type (TE235) cells overexpressing *rad3-N775* (pTE696), *rad3-N775-LZ* (pTE718), or empty vector (pTE101) and *rad3Δ* (TE890) cells with empty vector were grown on plates containing 10 mM HU for 3 d. Left, HU plate. Right, key. (B) Requirement of the P-site for Rad3-N775p dominant negative activity. Wild-type (TE235) cells expressing *rad3-N775* (pTE696), *rad3-N690* (pTE698), *rad3-N541* (pTE700), or *rad3-N775-P* (pTE710) were grown on plates containing 10 mM HU. Left, HU plate. Right, key. (C) Diagram of mutant Rad3p proteins. The leucine zipper and P-site are indicated by black boxes. Deletions of leucine zipper motif and P-site are indicated by a gap. A plus sign next to the protein indicates it can function as a dominant negative, and a negative sign indicates that it does not have this capability. (D) Expression of Rad3p N-terminal alleles. Anti-HA antibodies were used to Western blot extracts from cells expressing HA-tagged versions of the N-terminal proteins. Lane 1, HA-*rad3-N775* (pTE697); lane 2, HA-*rad3-N690* (pTE699); lane 3, HA-*rad3-N541* (pTE701); lane 4, HA-*rad3-N775-LZ* (pTE717); lane 5, HA-*rad3-N775-P* (pTE709).

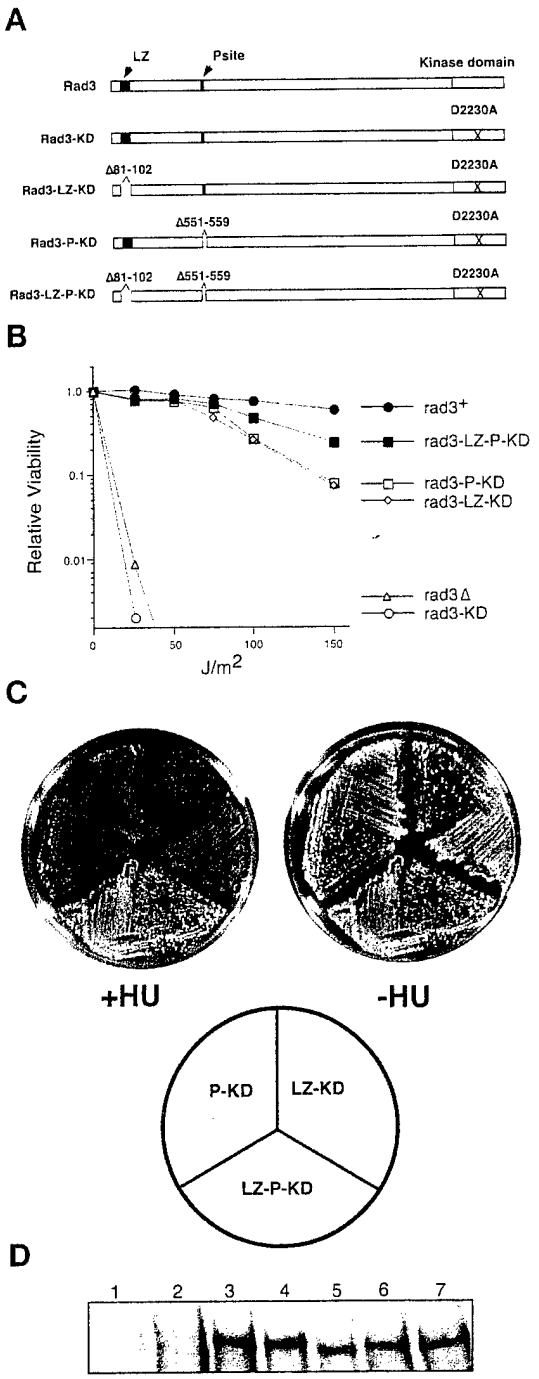

Figure 6. LZ and P-site mutations are additive. (A) Diagram of mutant Rad3 proteins. See Figures 1 and 4 for explanation of symbols. The position of the mutation (D2230A) is indicated with a black X. (B) The LZ and P mutations substantially reduce the dominant negative activity of the Rad3-KD allele. Relative viability after indicated doses of UV irradiation of *rad3*Δ cells (TE890) expressing empty vector (pTE101) and wild-type cells (TE235) overexpressing *rad3*$^+$ (pTE157), *rad3-KD* (pTE791), *rad3-LZ-KD* (pTE743), *rad3-P-KD* (pTE750), and *rad3-LZ-P-KD* (pTE745). The results shown are the average of two independent experiments. Note that the difference between the double and triple mutants is significant at 150 J/m^2. (C) The triple mutant Rad3-LZ-P-KD is a weaker dominant negative than the double mutants Rad3-LZ-KD and Rad3-P-KD on plates containing HU. Wild-type (TE235) cells overexpressing *rad3-LZ-KD*, *rad3-P-KD*, and *rad3-LZ-P-KD* were grown for 3 d on plates with (+HU) or without (−HU) 10 mM HU. (D) Expression of the Rad3p proteins. Rad3p polyclonal antibodies (HM126) were used to Western blot extracts made from *rad3*Δ cells transformed with rep3x vector control (lane 1); wild-type cells transformed with rep3x vector control (lane 2), rep1-*rad3*$^+$ (lane 3), rep1-*rad3-KD* (lane 4), rep1-*rad3-LZ-KD* (lane 5), rep1-*rad3-P-KD* (lane 6), and rep1-*rad3-LZ-P-KD* (lane 7). Note that endogenous Rad3p is not detected under these conditions (lane 2).

Additive Effects of LZ and P-Site Mutations

To ascertain whether the leucine zipper and the P-site affect the same or different functions, the dominant negative activity of *rad3-KD* constructs lacking either the leucine zipper or the P-site or both sites was examined. If these sites interact with the same protein, we would expect that deletion of both sites would have the same effect as deletion of one of the sites. If these sites interact with different proteins, the effects of deleting both might be additive.

To do these experiments we started with the *rad3-KD* allele and then deleted either the leucine zipper (*rad3-LZ-KD*), the P-site (*rad3-P-KD*), or both (*rad3-LZ-P-KD*) (Figure 6A). Mutation of either the leucine zipper or the P-site greatly reduced but did not completely abolish the dominant negative activity of the *rad3-KD* allele in both the UV (Figure 6B) and liquid HU assays (our unpublished results). However, deletion of both sites, *rad3-LZ-P-KD*, resulted in a further reduction of dominant negative activity. In the UV assay, the difference between the double and triple mutants was only modest, but is significant at the 150-J/m^2 time point. In the HU liquid assay, the triple mutant also reduced the dominant negative activity of the *rad3-KD* allele more than mutation of each site on its own, although the differences were small (our unpublished results). Therefore, to examine the HU response of the double and triple mutants, we used the plate assay, which requires the cells to grow for many generations in HU and thus is a more stringent test of dominant negative activity. Although cells overexpressing the *rad3-KD* allele grew poorly on plates (Bentley *et al.*, 1996; our unpublished results), cells overexpressing the double and triple mutants grow normally (Figure 6C), indicating that these mutations eliminate the toxicity of the *rad3-KD* allele as well as its dominant negative activity. In the presence of HU, the triple mutant *rad3-LZ-P-KD* clearly grows better than either of the double mutants (Figure 6C). All of these proteins are expressed at equal levels (Figure 6D). Thus, the leucine zipper and the P-site mutations work additively to reduce the dominant negative activity of *rad3-KD*, suggesting that the two sites could affect different Rad3p functions.

The Leucine Zipper and P-Site Are Required for Normal Function of Rad3p

Because deletion of the LZ and P-sites abrogates dominant negative activity, we wished to determine whether these sites are also required for the normal function of *rad3*$^+$. To do so, we deleted these sites from full-length *rad3*$^+$ (Figure 7A) and examined the ability of these alleles to complement a *rad3*Δ strain. Neither *rad3-LZ* nor *rad3-P* was able to complement the HU or UV sensitivity of a *rad3*Δ strain, indicat-

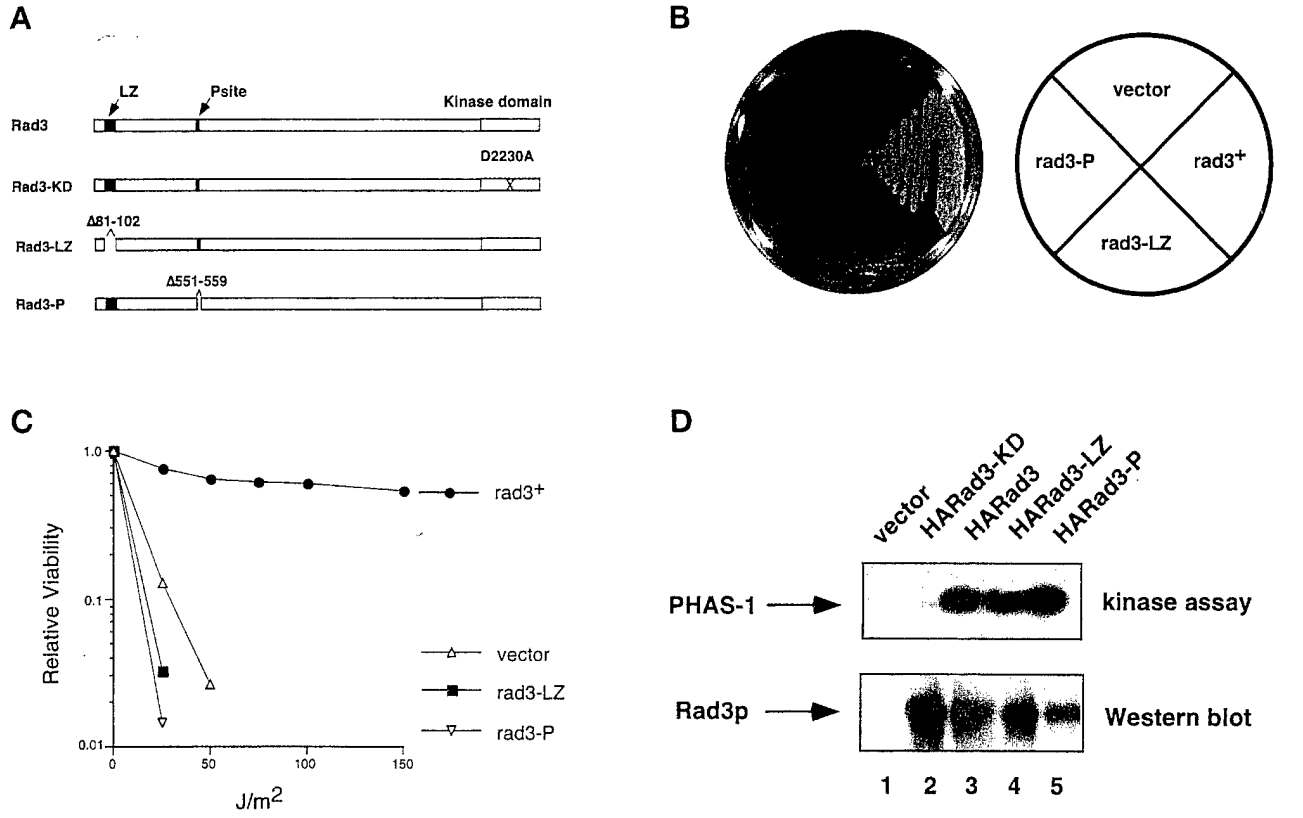

Figure 7. Leucine zipper and P-site are required for normal function of Rad3 protein. (A) Rad3 proteins are diagrammed approximately to scale. See Figure 5 for explanation of symbols. (B) *rad3*Δ (TE890) cells transformed with rep3x (pTE101), rep1-HA-*rad3*$^+$ (pTE541), rep1-HA-*rad3-LZ* (pTE716), or rep1-HA-*rad3-P* (pTE706) were grown on plates containing 10 mM HU. (C) Relative viability of the same strains after exposure to the indicated doses of UV irradiation. Results shown are the average of two independent experiments. (D) Rad3-LZp and Rad3-Pp are active kinases. Anti HA-immunopreciptates were prepared from *rad3*Δ (TE890) cells transformed with the plasmids indicated above. Half of each immunoprecipitate was assayed for PHAS-1 kinase activity, and the other half was subjected to SDS-PAGE, Western blotted, and probed with HA monoclonal antibody.

ing that the LZ and P-sites are indeed required for the normal function of *rad3*$^+$ (Figure 7, B and C). To determine whether either the leucine zipper or P-site is required for catalytic activity, the kinase activities of Rad3-LZp and Rad3-Pp were measured. Neither mutant had significantly diminished activity compared with wild-type protein (Figure 7D, lanes 3–5), and all three proteins phosphorylated significantly more PHAS-1 than a kinase-dead form of Rad3p (Figure 7D, lane 2). Because the mutated proteins are expressed well (Figure 7D, bottom panel) and retain catalytic activity, we believe that the structures of the proteins are grossly intact. These data indicate that these sites are not required for kinase activity of Rad3p and, moreover, that kinase activity is not sufficient for full Rad3p function.

The Leucine Zipper Is Not Required for Rad3p Self-Association or for Interaction with Chk1p

Having identified the putative leucine zipper as a probable protein–protein interaction site required for Rad3p function but not for kinase activity, we wished to further investigate the molecular mechanism of its function. Rad3p has been shown to self-associate (Bentley *et al.*, 1996). Because leucine zipper motifs have been shown to be important in mediating both hetero- and homodimerization (Phizicky and Fields, 1995), the self-association capabilities of Rad3 proteins lacking the leucine zipper motif were examined. HA- and myc-tagged forms of wild-type Rad3p and Rad3-LZp were constructed and cotransformed in various combinations into wild-type cells (strain TE236; see Table 1). In parallel experiments, either HA-Rad3p or myc-Rad3p was immunoprecipitated with the appropriate monoclonal antibody. Immunoprecipitations were resolved by SDS-PAGE and probed with both the HA and myc antibodies. As has been shown by others (Bentley *et al.*, 1996), Rad3p self-associates, because HA-Rad3p can be precipitated by anti-myc antibodies when coexpressed with myc-Rad3p (Figure 8A, lane 1) but was absent from immunoprecipitates from strains transformed with only the myc-tagging vector (Figure 8A, lane 6).

Using Rad3 proteins that contained deletions of the leucine zipper, we found that the leucine zipper is not required for Rad3p self-association. We were able to immunoprecipitate HA-Rad3-LZp with either myc-Rad3-LZp

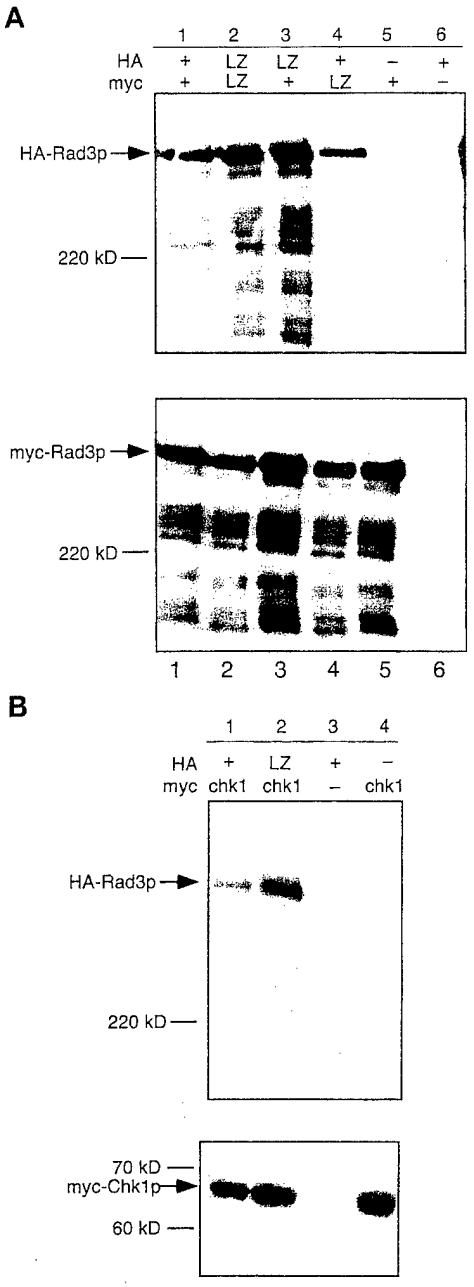

Figure 8. Rad3p leucine zipper motif is not required for self-association or for interaction with Chk1p. Wild-type cells (TE236) were cotransformed with the indicated *rad3* and/or *chk1* alleles, and myc immunoprecipitations were performed on extracts from the indicated strains. Samples were split, resolved on SDS-PAGE gels, blotted, and probed with either anti-HA antibodies (top) or anti-myc antibodies (bottom). (A) Self-association assay. Lane 1, rep41-HA-*rad3*$^+$ (pTE521) and rep42-myc-*rad3*$^+$ (pTE748); lane 2, rep41-HA-*rad3-LZ* (pTE746) and rep42-myc-*rad3-LZ* (pTE747); lane 3, rep41-HA-*rad3-LZ* and rep42-myc-*rad3*$^+$; lane 4, rep41-HA-*rad3*$^+$ and rep42-myc-*rad3-LZ*; lane 5, rep41-HAtag vector (control) (pTE119) and rep42-myc-*rad3*$^+$; lane 6, rep41-HA-*rad3*$^+$ and rep42-myctag vector (control) (pTE120). (B) Chk1p/Rad3p coimmunoprecipitation assay. Lane 1, rep41-HA-*rad3*$^+$ and rep42-myc-*chk1*$^+$ (pTE792); lane 2, rep41-HA-*rad3-LZ* and rep42-myc-*chk1*$^+$; lane 3, rep41-HA-*rad3* $^+$and rep42-myctag vector (control); lane 4, rep41-HAtag vector and rep42-myc-*chk1*$^+$.

(Figure 8A, lane 2) or wild-type myc-Rad3p (Figure 8A, lane 3). We were also able to immunoprecipitate wild-type HA-Rad3p with myc-Rad3-LZp (Figure 8A, lane 4). Similar results were obtained in the reciprocal experiment, immunoprecipitating with an HA antibody and then blotting for myc-Rad3p protein (our unpublished results). Thus, the leucine zipper motif is not required for self-association as measured by this assay.

S. pombe Rad3p has recently been shown to coimmunoprecipitate with Chk1p (Martinho *et al.*, 1998). We noticed a site in Chk1p that resembles a leucine zipper at amino acids 437–465 (Lx6Lx6Lx6Lx6K) and hypothesized that the putative leucine zipper in Rad3p may mediate its interaction with Chk1p. However, we were able to immunoprecipitate both wild-type HA-Rad3p and mutant HA-Rad3-LZp with myc-Chk1p (Figure 8B, lane 2), suggesting that the Rad3p leucine zipper motif is not required for the interaction observed in this assay.

DISCUSSION

Relatively little is known about the function of sequences outside the kinase domain in the PI3KR family of kinases. In this study, we have identified sites important for the normal cellular functions of *S. pombe* Rad3p, a member of this family. Our data indicate that sequences outside the kinase domain have at least two important functions. First, these sequences are required for catalytic activity. Second, we have identified two sites in the N terminus of Rad3p that may mediate interactions with regulators or substrates but are not required for catalytic activity.

The Isolated Rad3p Kinase Domain Is Catalytically Inactive and Does Not Complement rad3Δ Mutants

Our results demonstrate that the isolated Rad3p PI3-like-kinase domain is not sufficient for Rad3p function. Even the largest C-terminal fragment examined did not fully rescue the HU and UV sensitivities of *rad3Δ* cells (Figure 1). Furthermore, none of the C-terminal fragments exhibited kinase activity in our in vitro assay (Figure 2). The Rad3p kinase domain may require residues N-terminal to the kinase domain for catalytic activity for a variety of reasons. One possibility is that Rad3p kinase activity depends on Rad3p dimerization, mediated by sequences N-terminal to the kinase domain. Examples of this type of regulation include the receptor protein-tyrosine kinases, as well as some cytoplasmic protein-tyrosine kinases (for review, see Heldin, 1996). Indeed, Rad3p does self-associate, as shown by Bentley *et al.* (1996) and confirmed here (Figure 8), although it is not known whether this is necessary for catalytic activity. Alternatively, N-terminal sequences of Rad3p may function as an intramolecular positive regulator of the kinase domain. For example, they may be required to induce a catalytically active configuration or to stabilize interactions with substrates. Although we have not defined sequences outside the kinase domain required for catalytic activity, we have shown that the leucine zipper and the P-site are not required, as discussed below.

Our results are the first demonstration that sequences outside the kinase domain of any PI3KR kinase are required for catalytic or biological activity. In contrast to our findings, others have shown that a 390-amino-acid fragment of ATMp consisting of the isolated kinase domain is sufficient for

complementation of the radioresistant DNA synthesis and radiosensitivity phenotypes of A-T cells (Morgan *et al.*, 1997). In addition, the same ATMp fragment is capable of phosphorylating c-Abl without outside sequences (Baskaran *et al.*, 1997). However, in another study, this fragment was unable to phosphorylate replication protein A when transfected into A-T cells (Morgan and Kastan, 1997), suggesting that sequences outside the domain may be required in certain circumstances or for interactions with some substrates.

Because the isolated Rad3p kinase domain is not catalytically active, our results suggest that residues outside the kinase domain of Rad3p are more important for function than equivalent regions in ATMp. An interesting possibility is that the ATR and ATM PI3KR subgroups are structurally different and have distinct requirements for catalytic activity. Because the ATR subgroup exhibits regions of homology in the middle of the proteins, which are not present in the ATM subgroup (Savitsky *et al.*, 1995b; Bentley *et al.*, 1996), it is possible that these regions are required for the catalytic activity of proteins in the ATR subgroup, including Rad3p.

Another difference between the ATR and ATM-like PI3KR proteins is their sensitivity to the drug wortmannin. Our results indicate that Rad3p catalytic activity is relatively insensitive to wortmannin (Figure 2). The kinase activity of human ATRp is also relatively insensitive to the drug wortmannin (Sarkaria *et al.*, 1998). In contrast, the catalytic activity of human ATMp is inhibited by low concentrations of wortmannin (Banin *et al.*, 1998; Sarkaria *et al.*, 1998). Thus, these data further support the idea that Rad3p is more similar to human ATRp than ATMp.

It is also possible that differences in our experimental systems account for the requirement for non-kinase domain residues that we have observed. We have assayed Rad3p activity in strains harboring a large internal deletion in the *rad3* coding sequence (Bentley *et al.*, 1996). In contrast, the nature of the mutation in the *ATM* gene in the A-T cell line used by Morgan *et al.* (1997) is not known, leaving open the possibility of intragenic complementation, although the authors were careful to point out that they could not detect endogenous protein or message. Furthermore, functional complementation of A-T cells has proved problematic in the past; a number of rescuing clones were initially identified that did not map to the A-T locus, suggesting that suppression can occur by indirect mechanisms (Jongmans *et al.*, 1995; Shiloh, 1995; Ziv *et al.*, 1995). In contrast, no extragenic suppressors were isolated during the cloning of *rad3*$^+$ (Carr, unpublished observations), arguing that complementation of yeast mutants is a highly specific assay for *rad3* function. In light of our results, it will be interesting to examine the biological and catalytic activity of the isolated kinase domain of the newly identified fission yeast Tel1p (Naito *et al.*, 1998), which is more closely related to ATMp that Rad3p.

Use of Rad3p Dominant Negative Alleles to Identify Sequences Required for the Checkpoint Response

Sequences outside the kinase domain are required for Rad3p checkpoint function. To begin to understand the functions of nonkinase regions of Rad3p, we focused on the N terminus of Rad3p. Overexpression of N-terminal sequences of Rad3p causes wild-type cells to become checkpoint deficient (Figures 3 and 4), indicating that these sequences may be capable of sequestering proteins into nonfunctional complexes. Similarly, Morgan *et al.* (1997) demonstrated that expression of fragments of human ATMp, which contain a putative leucine zipper motif, caused dominant negative checkpoint phenotypes in a human tumor cell line. However, this study did not address what sites are required for these activities. To determine what sites are required for the dominant negative activity exhibited by overexpression of the Rad3-N775p, we performed deletion analysis (Figure 5). We found that the leucine zipper is necessary for the dominant negative activity of the N terminus. Interestingly, another site we call the P-site, located between amino acids 541 and 690 of the protein, is also necessary for dominant negative activity of the N terminus. Moreover, in the context of the full-length dominant negative allele *rad3-KD*, mutating both sites reduced dominant negative activity more than mutating either site alone (Figure 6), indicating that these sites are likely to be affecting two separate functions.

Importantly, both sites identified through dominant negative analysis are required for the normal cellular functions of Rad3p, because constructs deleted for either site fail to rescue *rad3*Δ cells (Figure 7). The failure of the mutants to complement *rad3*Δ cells is not due to destabilization of Rad3p, because these mutant proteins are expressed at levels comparable with wild type and retain full catalytic activity. This indicates that the gross structure of the Rad3 protein is likely to be intact, particularly because catalytic activity requires sequences outside the kinase domain. However, it is possible that the deletions create more localized disruptions of Rad3p structure.

We initially focused on the P-site because of its similarity to the PCNA binding motif found in a number of proteins from diverse organisms and considered the possibility that the P-site might mediate Rad3p binding to PCNA, the processivity factor for DNA polymerases. However, we have been unable to identify a P-site-dependent interaction between Rad3p and PCNA in preliminary studies (Chapman and Enoch, unpublished observations) and, moreover, have not identified PCNA binding sites in other proteins in the PI3KR family. Interestingly, *S. cerevisiae* Mec1p has a related site at an equivalent position, amino acids 537–545, **E**S**LL**S-**GILF** (conserved residues with P-site are bold; similar amino acid is underlined). Because this sequence is conserved in budding yeast but even less related to the PCNA binding site consensus, we believe that the P-site in Rad3p could be required for interactions with another protein that has yet to be identified. Possible candidates could include other fission yeast checkpoint proteins (see INTRODUCTION), particularly those which have *S. cerevisiae* homologues, such as Rad1p and Rad17p.

The putative leucine zipper domain of Rad3p is conserved in other PI3KR family members, but this is the first demonstration that the motif is functionally important. Leucine zippers frequently mediate homodimerization or heterodimerization with closely related proteins (Phizicky and Fields, 1995). However, surprisingly, we found that the putative leucine zipper is not required for self-association of Rad3p. Furthermore, it is not required to interact with Chk1p (Figure 8). It is possible that the coimmunoprecipitation assay we used (Bentley *et al.*, 1996), which uses overexpressed proteins, may not be sufficiently sensitive to detect differences in binding between the wild-type and mutant proteins. Alternatively, other sequences that have yet to be

identified may mediate Rad3p self-association and association with Chk1p, and the leucine zipper could be required for interactions with other proteins that have leucine zipper motifs. One interesting candidate for an interaction partner is the newly identified fission yeast Tel1p (Naito *et al.*, 1998), which has a leucine zipper-like site. Because putative leucine zippers are found in other members of the PI3KR family, interactions directed by this motif are likely to be an important aspect of regulation of this important class of kinases.

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APPENDIX III.

Chapman, C. R. and Enoch, T. (2000) Genetic interactions link fission yeast *rad3+* to DNA replication. *in preparation.*

Abstract

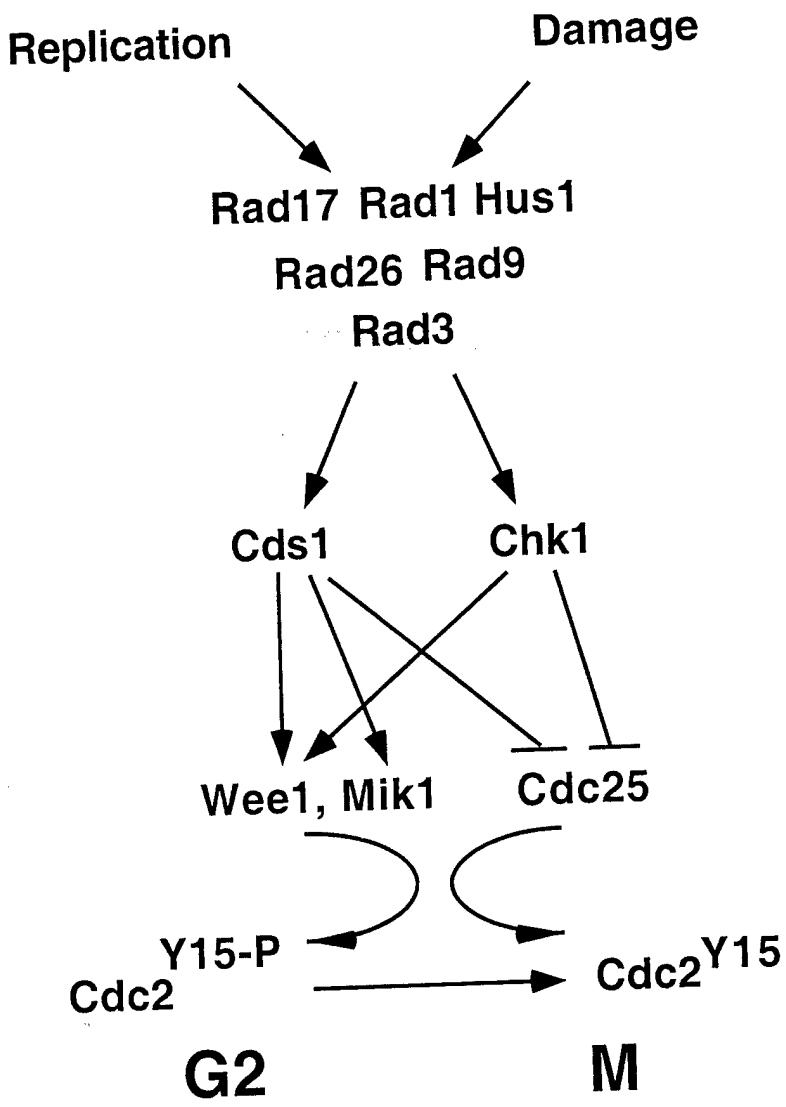
The fission yeast *rad3*+ checkpoint gene acts to delay cell division in the presence of incomplete DNA replication or DNA damage. *rad3*+ also acts to slow DNA replication in the presence of DNA damaging agents. Thus, DNA replication genes are candidates to be both activators and targets of eukaryotic checkpoint genes. To identify potential interactions between *S. pombe rad3*+ and DNA replication genes, we screened for synthetic dosage phenotypes by overexpressing *rad3*+ in a collection of DNA replication mutants. We have discovered a gene and allele-dependent interaction between *rad3*+ and *cdc6*, which encodes pol∂, the processive DNA polymerase. When *rad3*+ is overexpressed in two temperature sensitive *cdc6* mutants, *cdc6-23* and *pol∂ts1*, the cells elongate and arrest the cell cycle at the permissive temperature. Furthermore, these cells have an increased requirement for an intact mitotic checkpoint pathway. These data suggest that overexpression of *rad3*+ may interfere with DNA replication in these cells. In addition, we show that overexpression of *rad3*+ inhibits resumption of DNA replication in cells recovering from hydroxyurea-mediated S phase arrest. Both of these overexpression phenotypes can also be mediated by overexpression of kinase dead alleles of *rad3*, suggesting that catalytic activity is not required for overexpression of *rad3*+ to interfere with DNA replication. These two new overexpression phenotypes strongly suggest that *rad3*+ can influence DNA replication in the cell.

Introduction

Eukaryotic checkpoint pathways act to maintain the proper order of cell cycle events (Hartwell and Weinert, 1989). The S-M checkpoint in *S. pombe* ensures that mitosis (M phase) does not take place until DNA replication (S phase) is finished. In fission yeast, the S-M checkpoint pathway blocks cell cycle progression by downregulating the activity of the cyclin-dependent kinase Cdc2p, which promotes entry into mitosis (Enoch and Nurse, 1990; Enoch *et al.*,. 1991; Rhind *et al.*, 1997). Thus, certain *cdc2* mutants, such as *cdc2-3w* cells, fail to arrest the cell cycle in response to incomplete DNA replication (Enoch and Nurse, 1990). Although the Cdc2p kinase is regulated by multiple mechanisms, including requisite binding to a cyclin partner, the checkpoint pathway regulates Cdc2p by affecting the phosphorylation state of its tyrosine 15 residue (Rhind *et al.*, 1997). Cdc2p is only active when this residue is dephosphorylated (Gould and Nurse, 1989). The Cdc25p phosphatase (Russell and Nurse, 1986), which dephosphorylates and activates Cdc2p, and the Wee1p and Mik1p kinases (Russell and Nurse, 1987; Lee *et al.*, 1994; Lundgren *et al.*, 1991), which phosphorylate Cdc2p, are all targets of the checkpoint pathway (see Figure 1) (Furnari *et al.*, 1997; O'Connell *et al.*, 1997; Boddy *et al.*, 1998; Zeng *et al.*, 1998; Furnari *et al.*, 1999).

A number of fission yeast checkpoint genes function upstream of the *cdc25*$^+$, *wee1*$^+$, and *mik1*$^+$ cell cycle regulators. The *checkpoint rad* genes of *S. pombe*, which include *rad3*$^+$, *hus1*$^+$, *rad1*$^+$, *rad9*$^+$, *rad17*$^+$, and *rad26*$^+$, share similar mutant phenotypes and participate in both the S-M checkpoint and the G2-M DNA damage checkpoint, which blocks mitosis in the presence of DNA damage (Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Al-

Figure 1. Model of the *S. pombe* DNA replication and DNA damage checkpoint pathways. A number of fission yeast checkpoint proteins respond to blocked DNA replication or DNA damage. Rad1p, Rad9p, Rad17p, Rad26p, and Hus1p are believed to function upstream in the pathway, perhaps in sensing or detecting abnormal DNA structures. In the presence of blocked DNA replication or DNA damage, the Rad3p kinase phosphorylates Cds1p or Chk1p, respectively. In turn, these kinases regulate the Cdc25p phosphatase, and the Wee1p and Mik1p kinases, which control the inhibitory phosphorylation at tyrosine 15 (Y15) on Cdc2p. Cells cannot proceed into mitosis unless Cdc2p is dephosphorylated on Y15.


Replication
Damage
Rad17 Rad1 Hus1
Rad26 Rad9
Rad3
Cds1
Chk1
Wee1, Mik1
Cdc25
Y15-P
Cdc2
Cdc2Y15
G2
M

Khodairy *et al.*, 1994; Rowley *et al.*, 1992). *rhp9*$^{+}$/*crb2*$^{+}$ is another gene required for both checkpoints (Willson *et al.*, 1997; Saka *et al.*, 1997). The proteins encoded by these genes may be involved in monitoring incomplete DNA replication and/or DNA damage. In the presence of DNA damage or blocked DNA replication, the checkpoint kinase Rad3p is thought to send a checkpoint signal through the downstream kinases Chk1p and Cds1p (Walworth *et al.*, 1993; Murakami and Okayama, 1995; Walworth and Bernards, 1996; Lindsay *et al.*, 1998; Martinho *et al.*, 1998), which in turn control the cell cycle regulators Cdc25p, Wee1p, and Mik1p (see Figure 1) (Furnari *et al.*, 1997; O'Connell *et al.*, 1997; Boddy *et al.*, 1998; Zeng *et al.*, 1998; Furnari *et al.*, 1999).

Rad3p is believed to play a central role in the checkpoint pathway, in part because several checkpoint proteins, including Cds1p, Chk1p, Hus1p, are phosphorylated in a Rad3p-dependent manner (Walworth and Bernards, 1996; Kostrub *et al.*, 1997; Lindsay *et al.*, 1998; Martinho *et al.*, 1998). Rad3p is a member of a family of structurally and functionally related kinases from a number of diverse organisms (for reviews, see Hoekstra, 1997; Keith and Schreiber, 1995). Other members of this family of proteins include human ATRp and ATMp (Savitsky *et al.*, 1995a; Savitsky *et al.*, 1995b; Bentley *et al.*, 1996; Cimprich *et al.*, 1996), *S. cerevisiae* Mec1p (Weinert, 1992; Kato and Ogawa, 1994; Weinert *et al.*, 1994) and Tel1p (Greenwell *et al.*, 1995; Morrow *et al.*, 1995). Recently, *S. pombe* Tel1p was also identified (Naito *et al.*, 1998). Loss of *ATM* function in humans causes ataxia-telangiectasia, a disease in which patients suffer from a variety of symptoms, including a predisposition to cancer (for review, see Meyn, 1995). The large proteins (all greater than 200 kD) of this family each contain a C-terminal kinase domain that exhibits similarity to those of PI3

(phosphatidylinositol 3-) kinases, which phosphorylate lipids as well as proteins. Despite this similarity, the members of this PI3KR (PI3 kinase-related) family are only known to phosphorylate proteins. Many of the PI3KR proteins also possess putative leucine zipper motifs, which may mediate protein-protein interactions.

Although kinase activity is known to be required for Rad3p function (Bentley *et al.*, 1996), sequences N-terminal to the kinase domain are also necessary for proper checkpoint control (Chapter 2). Indeed, the isolated kinase domain does not have kinase activity *in vitro* and cannot complement a *rad3Δ* strain. In addition, deletion of the putative leucine zipper in the N-terminus of *rad3* abolishes checkpoint function of Rad3p without affecting kinase activity of the protein (see Chapter 2). N-terminal sequences may be required for Rad3p to interact with other proteins, and these interactions may be necessary for a proper response to checkpoint signals.

It is still unclear what DNA, protein, or DNA/protein structures activate the *checkpoint rad* genes, and initiate the checkpoint cascade. DNA replication complexes are excellent candidates for initiating the checkpoint signal. Mutation of a number of genes involved in the initiation of DNA replication abolishes the dependence of cell division on the completion of S phase, as these mutants enter mitosis in the absence of DNA replication initiation. In *S. pombe*, such genes include *orp1*$^+$/*cdc30*$^+$ (Grallert and Nurse, 1996) and *orp2*$^+$ (Leatherwood *et al.*, 1996), which are subunits of the Origin Recognition Complex; *polα*, polα primase (D'Urso *et al.*, 1995; Bhaumik and Wang, 1998); *rfc2*$^+$, a component of replication factor C (Reynolds *et al.*, 1999); and *rad4*$^+$/*cut5*$^+$, also believed to be involved with DNA replication (Saka and Yanagida, 1993). It is possible that some or all of these proteins are directly involved in activating the checkpoint

cascade. However, since DNA replication does not take place in these mutants, another alternative is that the structures which activate the *checkpoint rad* genes fail to form. In contrast, fission yeast genes involved in processive DNA replication, such as *pcn1*$^+$ (PCNA), subunits of polymerase ∂, and polymerase ε, do not appear to be required for checkpoint control because deletion of these genes leads to the cell division cycle (*cdc*) arrest phenotype (Waseem *et al.*, 1992; Francesconi *et al.*, 1993; MacNeill *et al.*, 1996; D'Urso and Nurse, 1997). However, in *S. cerevisiae*, certain C-terminal mutations in DNA polε abolish proper checkpoint activation (Navas *et al.*, 1995; Navas *et al.*, 1996; Dua *et al.*, 1998). Therefore, to understand how a checkpoint signal cascade initiates, it will be important to clarify the relationship between DNA replication genes and checkpoint genes.

Although DNA replication genes are candidates to be activators of the checkpoint pathway, they may also be targets of the checkpoint pathway. In humans, *S. cerevisiae*, and *S. pombe*, checkpoint genes are known to slow DNA synthesis in response to DNA damaging agents (Painter and Young, 1980; Paulovich and Hartwell, 1995; Lindsay *et al.*, 1998; Rhind and Russell, 1998). Human cells with mutations in *ATM* exhibit "radioresistant DNA synthesis" (RDS), the failure to slow DNA replication in the presence of DNA damaging agents (Painter and Young, 1980). The *MEC1* gene in *S. cerevisiae* is also known to function in an intra-S phase checkpoint that slows DNA synthesis in the presence of DNA damaging agents, such as methylmethane sulfonate (MMS) (Paulovich and Hartwell, 1995). *S. pombe rad3*$^+$ and *cds1*$^+$ also mediate a damage-induced S phase delay (Lindsay *et al.*, 1998; Rhind and Russell, 1998). Intriguingly, two groups recently found evidence for involvement of the *S.*

cerevisiae checkpoint proteins in inhibiting late-firing origins in the presence of either HU (hydroxyurea, which inhibits DNA replication) (Santocanale and Diffley, 1998) or MMS (Shirahige *et al.*, 1998). Thus, checkpoint proteins may regulate the rate of DNA synthesis by influencing the activities of DNA replication proteins.

Although fission yeast *rad3Δ* mutants are viable, its *S. cerevisiae* homologue, *MEC1,* is an essential gene. The essential function of *MEC1* appears to involve regulation of the rate of DNA synthesis, since nucleotide availability may be rate-limiting for DNA synthesis in *S. cerevisiae* (Desany *et al.*, 1998). For example, at least two classes of mutations allow *mec1Δ* mutants to survive. One type of suppression involves second-site mutations in genes involved in the initiation of DNA replication, such as *CDC7* and *DBF4* (Desany *et al.*, 1998). Presumably, these mutations compensate for a lack of *MEC1* function by slowing down origin firing. Another class of *mec1Δ* suppressors increases the availability of nucleotides in the cell. This class includes overexpression of *RNR1*, a subunit of ribonucleotide reductase (Desany *et al.*, 1998) and deletion of *SML1,* an inhibitor of ribonucleotide reductase (Zhao *et al.*, 1998). Thus, the essential function of *MEC1* may be to coordinate DNA replication with nucleotide availability. Although *rad3*$^+$ is not essential, it may also be involved in regulating the rate of DNA replication when nucleotide availability is low.

In this study, we used genetic approaches to investigate the relationship between the fission yeast checkpoint kinase *rad3*$^+$ and DNA replication genes. We show that overexpressed *rad3*$^+$ can interfere with DNA replication in situations where DNA replication may already be compromised. Using a genetic method known as synthetic dosage lethality (Kroll *et al.*, 1996), we have

discovered a specific allele-dependent genetic interaction between *rad3*$^+$ and *cdc6*, which encodes polδ, the processive DNA polymerase (Nasmyth and Nurse, 1981; Pignede *et al.*, 1991; Park *et al.*, 1993; Francesconi *et al.*, 1993; Iino and Yamamoto, 1997). Overexpression of *rad3*$^+$ exacerbates the phenotype of two out of five alleles of *cdc6*, such that an intact checkpoint pathway becomes required at the permissive temperature. Our data suggest that overexpression of *rad3*$^+$ interferes with DNA replication in these cells. We also show that overexpression of *rad3*$^+$ can inhibit resumption of DNA replication in wild-type cells recovering from hydroxyurea-mediated S-phase arrest. This extension of S phase exhibited by cells overexpressing *rad3*$^+$ is dependent on *chk1*$^+$. Both of these overexpression phenotypes can also be mediated by catalytically inactive forms of *rad3*, suggesting that Rad3p kinase activity is not required for *rad3*$^+$ overexpression to interfere with DNA replication in these situations. These results support the idea that Rad3p regulates DNA replication in response to DNA replication blocks. Models which might explain the mechanism behind the *rad3*$^+$ overexpression phenotypes are considered.

Results

rad3$^+$ exhibits a specific synthetic dosage genetic interaction with *cdc6* (*polδ*).

We took a genetic approach to investigate possible interactions between the *S. pombe* checkpoint gene *rad3*$^+$ and DNA replication genes. Synthetic lethality occurs when the combination of mutations in two independent genes, neither of which are essential for viability on their own, results in cell death. A

synthetic lethal interaction between two genes suggests that the genes may participate in a common function in the cell. However, we reasoned that in this case, synthetic lethality would not be appropriate for screening for specific interactions between *rad3*+ and DNA replication genes, since checkpoint function generally becomes essential when DNA replication is compromised.

Therefore, to identify specific interactions between *rad3*+ and DNA replication genes, we used a variation of synthetic lethality, called synthetic dosage lethality. Synthetic dosage lethality, developed by Kroll *et al.* (1996), is similar to synthetic lethality, but instead of investigating the effects of combining two mutations, one combines overexpression of one gene with mutation of another. An enhanced phenotype may suggest a physical or functional relationship between the overexpressed and mutant genes. For example, Kroll *et al.* found that in *S. cerevisiae*, overexpression of *CTF13*, a centromere binding protein, caused synthetic dosage lethality in chromosome segregation mutants, but not in DNA replication mutants; on the other hand, *ORC6*, a subunit of the origin recognition complex, caused synthetic dosage lethality only in DNA replication mutants (Kroll *et al.*, 1996). Thus, by identifying synthetic dosage interactions, one can gain evidence that two genes may have interrelated functions in the cell. To identify interactions between *rad3*+ and DNA replication genes, we overexpressed *rad3*+ in various DNA replication mutants, and screened for synthetic dosage lethality.

To screen for synthetic dosage interactions, we employed a vector in which *rad3*+ expression is controlled by the thiamine-repressible *nmt1*+ promoter (Bentley *et al.*, 1996)(see Table 7 for plasmids used in this study). By

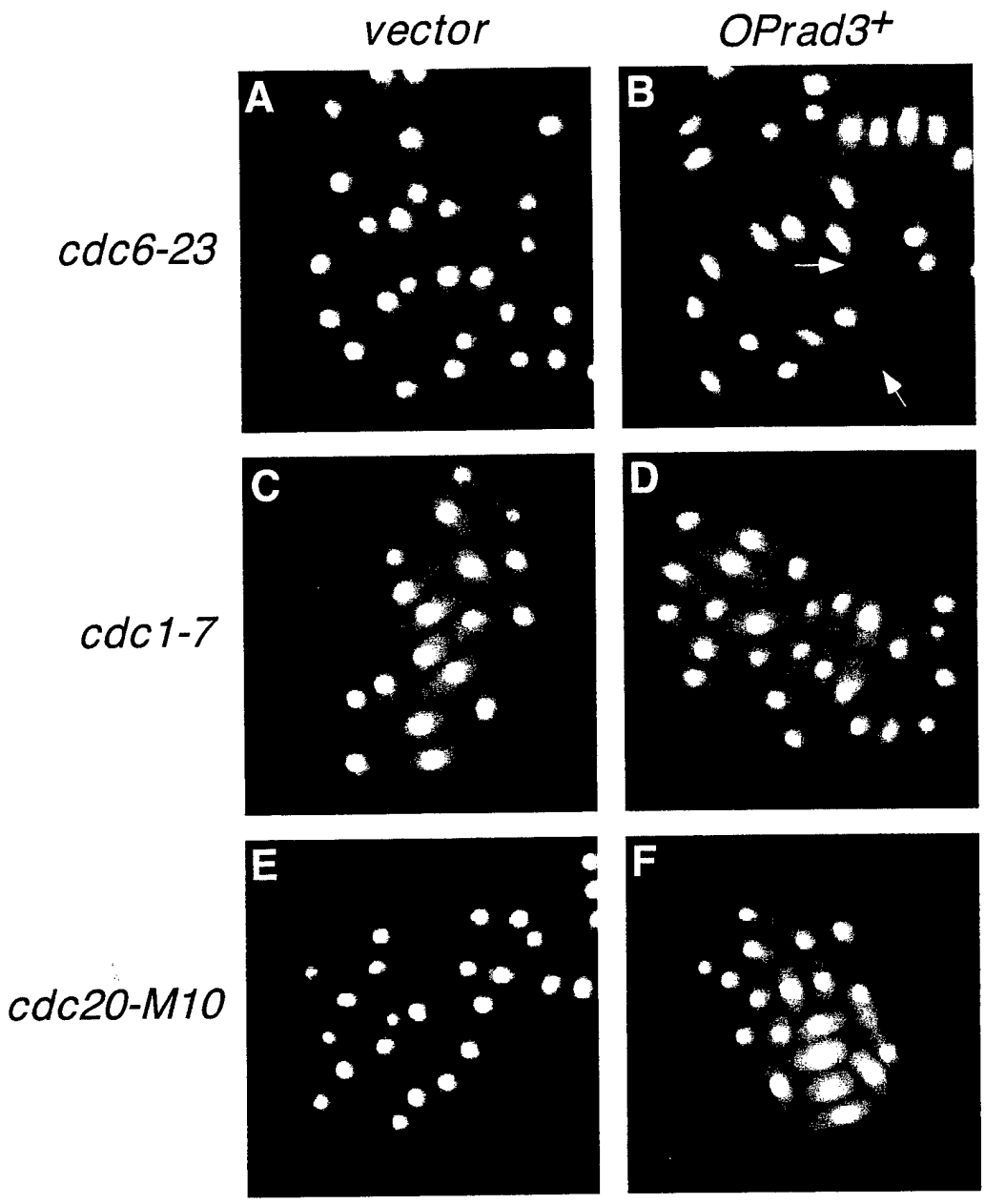
removing thiamine from the media, gene expression from this promoter can be induced approximately 80 fold (Maundrell, 1990). We transformed the rep3x control vector and the rep1-*rad3*$^+$ construct into a variety of temperature sensitive DNA replication mutant strains, including *cdc6-23*, *cdc1-7* and *cdc20-M10* (see Table 6 for strains used in this study). *cdc6*$^+$ encodes the DNA polymerase ∂ catalytic subunit (Pignede *et al.*, 1991; Park *et al.*, 1993; Francesconi *et al.*, 1993; Iino and Yamamoto, 1997), and *cdc1*$^+$ encodes a regulatory subunit of polδ (MacNeill *et al.*, 1996). *cdc20*$^+$ encodes the DNA polymerase ε catalytic subunit (D'Urso and Nurse, 1997). At their nonpermissive temperatures of 37°C, all of these DNA replication mutants accumulate as long cells arrested in the cell cycle (Nurse *et al.*, 1976; Nasmyth and Nurse, 1981), since they cannot complete DNA replication but their checkpoints remain intact. However, at the permissive temperature of 25°C, these DNA replication mutants do not arrest, but rather grow normally.

To investigate possible synthetic dosage interactions, single colony transformants were grown at 25°C in starter cultures containing thiamine to keep the *nmt1*$^+$ promoter in the repressed state. Thiamine was then washed out of the media, and cells were inoculated into minimal media lacking thiamine and grown under inducing conditions. Cultures were diluted after approximately 24 hours to maintain exponential growth. After 45-48 hours, cells were fixed with ethanol and stained with the DNA-dye DAPI to examine the length of the cells. As shown in Figure 2A and 2B, *cdc6-23* cells overexpressing *rad3*$^+$ (*OPrad3*$^+$ *cdc6-23)* cells accumulated as long, cell cycle arrested cells, while *cdc6-23* cells with vector alone were of relatively normal cell size. In contrast, *cdc1-7* and

cdc20-M10 cells overexpressing *rad3*$^+$ were similar in size to their respective vector controls (Figure 2C-F). Furthermore, wild-type cells expressing *rad3*$^+$ appear slightly smaller than vector controls (see Figure 3G and H). We do not believe that the synthetic phenotype caused by *rad3*$^+$ overexpression in *cdc6-23* cells is due to an inactivation of the DNA replication checkpoint pathway, since the cells exhibit a classic *cdc* arrest phenotype. This cell cycle arrest phenotype exhibited by *OPrad3*$^+$ *cdc6-23* cells indicates that the cells are growing, but not dividing, suggesting that cell cycle progression is arrested.

This synthetic dosage phenotype was also observed by examining cell growth on plates at 25°C. We transformed the rep3x control vector and the rep1-*rad3*$^+$ construct into four additional DNA replication mutant strains, including *cdc27-M57*, *cdc27-P11*, *cdc18-K46*, and *cdc19-P1*. *cdc27*$^+$ encodes a DNA polymerase ∂ subunit (Hughes *et al.*, 1992, Hughes *et al.*, 1999; MacNeill *et al.*, 1996). *cdc18*$^+$ and *cdc19*$^+$ encode an ORC binding protein and an MCM protein, respectively (Kelly *et al.*, 1993; Muzi Falconi *et al.*, 1996; Forsburg and Nurse, 1994). Single colony transformants were streaked onto minimal medium plates containing thiamine to keep the *nmt1*$^+$ promoter in its repressed state. After the colonies had grown, the plates were replica plated to minimal medium plates lacking thiamine. After 3 days of growth under inducing conditions, the morphology of the cells was observed microscopically. *cdc6-23* cells expressing *rad3*$^+$ (*OPrad3*$^+$ *cdc6-23* cells) were clearly longer than *cdc6-23* cells

Figure 2. Overexpression of *rad3*$^+$ exhibits a specific synthetic dosage genetic interaction with *cdc6*, which encodes the catalytic subunit of DNA polymerase ∂. Various DNA replication mutants were transformed with the rep3x vector (pTE101) or the rep1-*rad3*$^+$ plasmid (pTE157), which directs expression of *rad3*$^+$ from the thiamine-repressible *nmt1*$^+$ promoter. Starter cultures of single colony transformants were grown in EMM media containing thiamine; the cells were subsequently inoculated into thiamine-free medium to induce expression from the *nmt1*$^+$ promoter, and allowed to grow exponentially for 45-48 hours. Cells were ethanol-fixed, and stained with the DNA-dye DAPI. Photomicrographs are shown of *cdc6-23* cells (TE697) expressing the rep3x vector control (pTE101) (A) or *rad3*$^+$ (pTE157) (B); *cdc1-7* cells (TE699) expressing the vector (C) or *rad3*$^+$ (D); *cdc20-M10* (TE877) cells expressing the vector (E) or *rad3*$^+$ (F). Arrows indicate examples of elongated cells.


vector
OPrad3+
A
B
cdc6-23
C
D
cdc1-7
E
F
cdc20-M10

expressing the rep3x vector controls (Table 1). However, in the other DNA replication mutants, cells expressing *rad3*$^+$ were not longer than the vector controls. From these data, we conclude that *rad3*$^+$ exhibits a gene-specific synthetic dosage interaction with *cdc6*, which encodes the leading strand DNA polymerase ∂.

The interaction between *rad3*$^+$ and *cdc6* (pol∂) is allele-dependent.

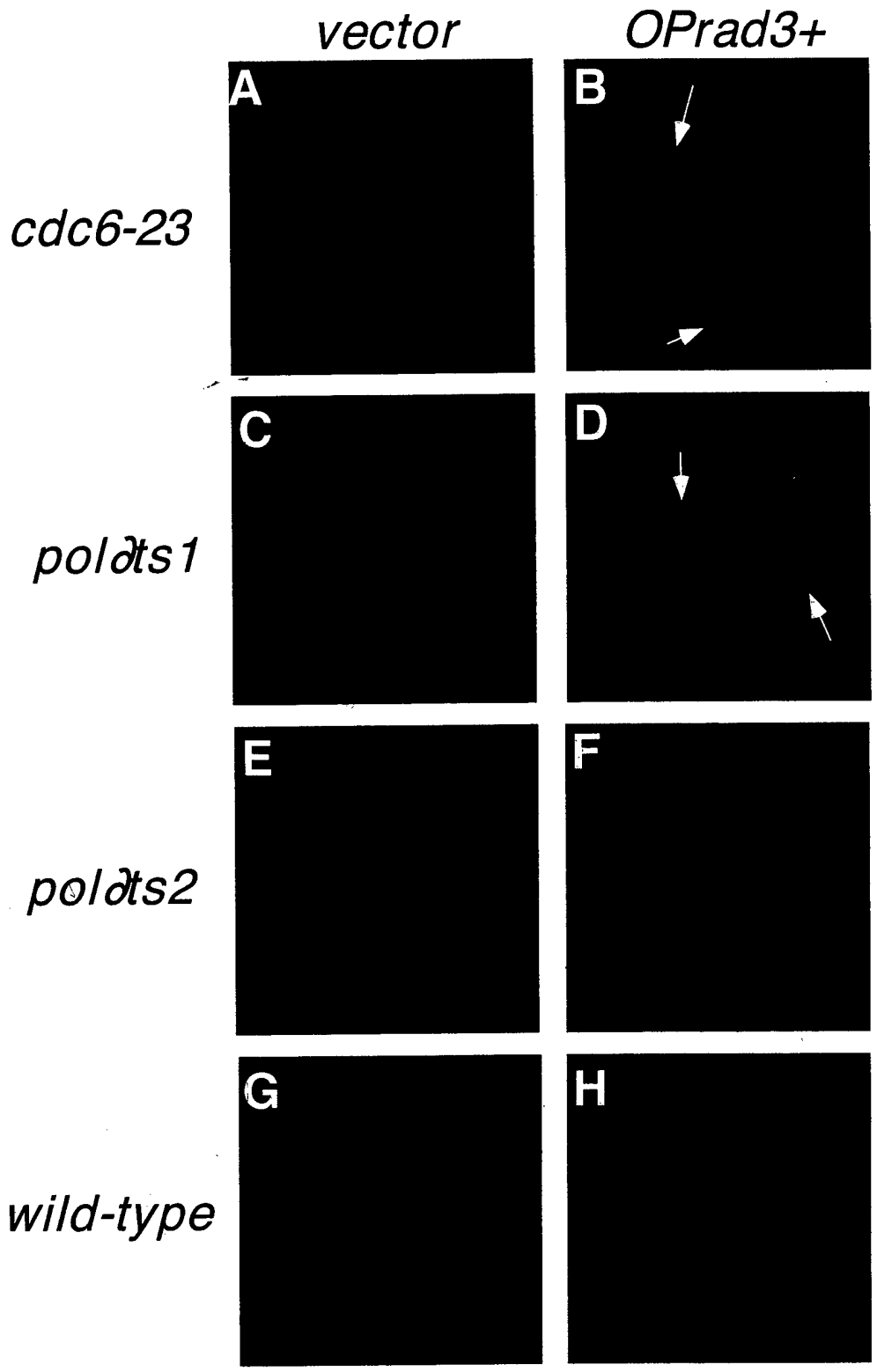
To further our understanding of the nature of the interaction between *rad3*$^+$ and *cdc6*, we wished to examine whether the synthetic phenotype was the result of a general loss of *cdc6* activity or whether the effect depended on mutation of a specific function or domain of *cdc6*. Therefore, we investigated the ability of overexpression of *rad3*$^+$ to cause a synthetic phenotype in additional alleles of *cdc6*, including *pol∂ts1* and *pol∂ts2*. We also examined the effects of overexpression of *rad3*$^+$ in wild-type cells. To do this, we grew the cells in liquid culture as described above. As shown in Figure 3, overexpression of *rad3*$^+$ caused the synthetic dosage phenotype in *cdc6-23* and *pol∂ts1* mutants, but did not cause elongation in *pol∂ts2* mutants. Wild-type cells overexpressing *rad3*$^+$ actually appeared smaller than vector controls (Figure 3). We also scored the phenotype of overexpressing *rad3*$^+$ in two additional *cdc6* alleles, *cdc6-121* and *pol∂ts3*, by examining the growth of cells on plates at 25°C. Overexpression of *rad3*$^+$ in *cdc6-121* and *pol∂ts3* did not cause elongation of the cells (data not shown). Thus, only two out of five of the *cdc6* alleles tested exhibit a synthetic dosage interaction with *rad3*$^+$. The allele dependence of the synthetic dosage

Table 1. *rad3*$^{+}$ exhibits a specific genetic interaction with *cdc6*, or polδ, the processive DNA polymerase.

Gene/Allele (Strain)	Function	*OPrad3*$^{+}$ (25°C)
cdc6-23	polδ catalytic subunit	*cdc*[a]
cdc1-7	polδ regulatory subunit	--[b]
cdc27-M57	polδ regulatory subunit	--
cdc27-P11	"	--
cdc20-M10	polε regulatory subunit	--
cdc18-K46	ORC binding protein	--
cdc19-P1	MCM protein	--

[a] *cdc* indicates that cells exhibit cell division cycle arrest (elongated) phenotype.
[b] -- indicates that the cell lengths are similar to vector controls.

Figure 3. The interaction between *rad3*+ and *cdc6* is allele-dependent. Cells expressing vector controls or rep1-*rad3*+ were inoculated into thiamine-free medium to induce expression from the *nmt1*+ promoter and grown for 45-48 hours. The cells were then fixed and DAPI-stained, as described in Figure 2. Photomicrographs of *cdc6-23* cells (TE697) expressing rep3x vector (pTE101) (A) and *rad3*+ (pTE157) (B) are shown, as well as *pol∂ts1* cells (TE905) expressing vector (C) and *rad3*+ (D), *pol∂ts2* cells (TE907) expressing vector (E) and *rad3*+ (F), and wild-type cells (TE235) expressing vector (G) and *rad3*+ (H). Arrows indicate examples of elongated cells.


vector
OPrad3+
A
B
cdc6-23
C
D
polδts1
E
F
polδts2
G
H
wild-type

phenotype between *rad3*$^+$ and *cdc6* indicates that the genetic interaction is quite specific and may be related to a particular function of *cdc6*$^+$.

Other checkpoint genes and *rad3* alleles do not exhibit an interaction with *cdc6*.

If *cdc6-23* cells are sensitive to a general increase in checkpoint function, then the synthetic phenotype exhibited by *OPrad3*$^+$ in *cdc6-23* cells might be mimicked by overproducing other checkpoint genes. Alternatively, the effect may be specific to *rad3*$^+$. To determine if other checkpoint genes cause elongation in *cdc6-23* cells at the permissive temperature of 25°C, various checkpoint genes under the control of the *nmt1*$^+$ promoter were transformed into *cdc6-23* cells. We examined the morphology of cells on plates under inducing conditions to score the phenotypes. None of the other *checkpoint rad* genes, including *rad1*$^+$, *rad9*$^+$, *rad17*$^+$, *rad26*$^+$, and *hus1*$^+$ appeared to exhibit a specific interaction with *cdc6* (see Table 2), although interpretation of the data is complicated because overexpression of some of the checkpoint genes causes phenotypes in wild-type cells. For example, overexpression of *hus1*$^+$ and *rad26*$^+$ partially inhibit growth in wild-type cells, and overexpression of *rad17*$^+$ causes wild-type cells to slightly elongate (data not shown, and Forbes *et al.*, 1998; Kostrub *et al.*, 1998). However, we did not observe any novel phenotypes, or additional elongation, when these genes were overexpressed in *cdc6-23* cells. rep1-*rad1*$^+$ and rep1-*rad9*$^+$ also failed to cause elongation in *cdc6-23* cells. The effects of overexpressing *cds1*$^+$ or *chk1*$^+$ were uninterpretable since they cause a cell cycle delay when overexpressed in wild-type cells (Ford *et al.*, 1994; Forbes

Table 2. Other checkpoint genes and *rad3* alleles do not exhibit an interaction with *cdc6* (polδ).

Overexpressed Gene/Allele	*cdc6 permissive temp*
rep3x (vector)	--[a]
rad3+	cdc[b]
rad3-N775	–
rad3-C725	–
rad3-LZ	cdc
hus1+	–
rad1+	–
rad9+	–
rad17+	–
rad26+	–

[a]-- indicates that the cell lengths are similar to vector controls.
[b]*cdc* indicates that cells exhibit cell division cycle arrest (elongated) phenotype.

et al., 1998). Thus, *rad3*$^+$ is the only checkpoint gene, of the ones we examined, that is capable of causing a specific cell cycle arrest phenotype in *cdc6-23* cells at the permissive temperature.

Rad3p kinase activity is not required for the synthetic dosage phenotype with *cdc6*.

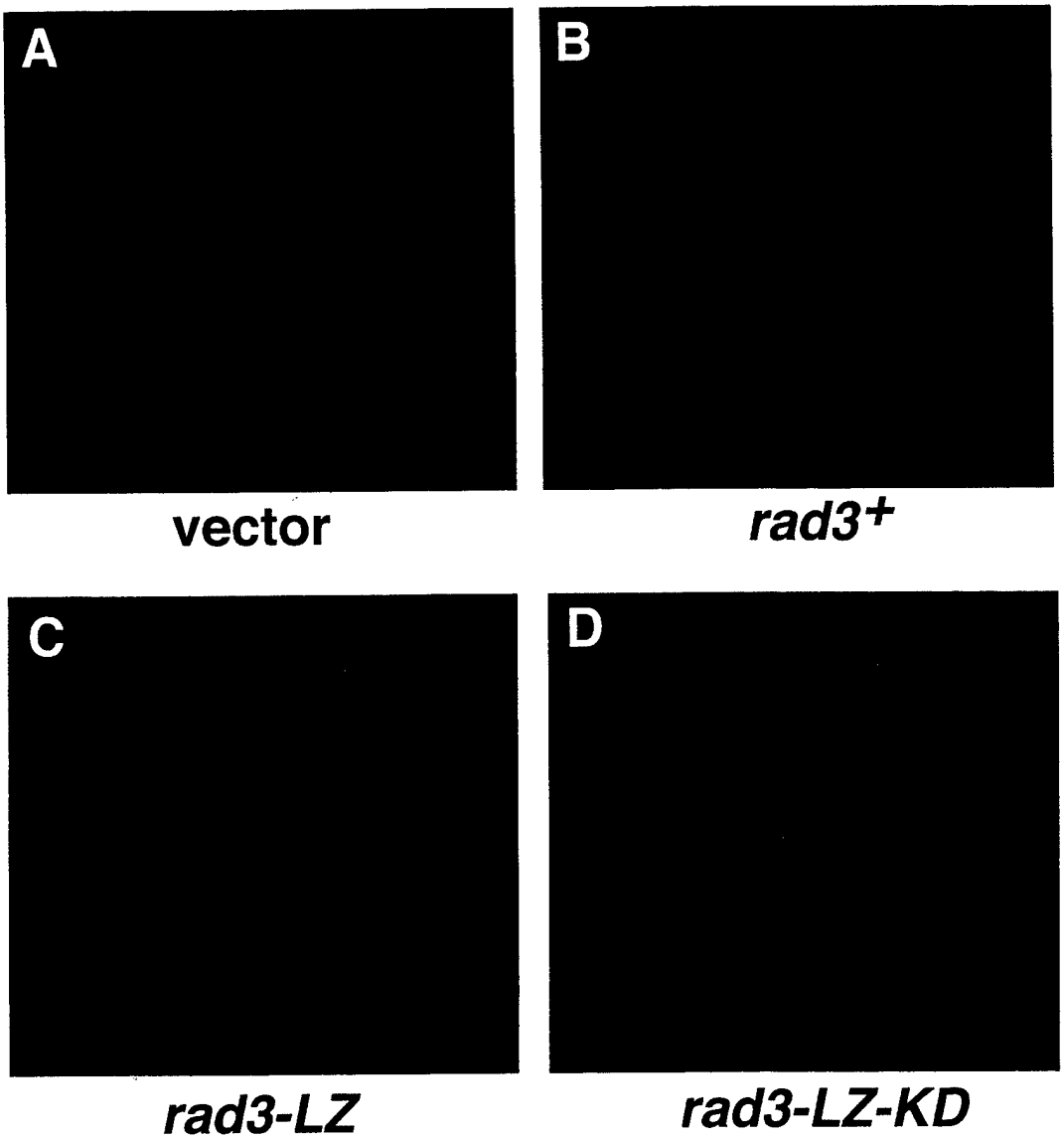
To further elucidate the mechanism of the synthetic dosage interaction between *rad3*$^+$ and *cdc6*, we wished to determine which structural elements of Rad3p are required for the synthetic dosage phenotype. For example, checkpoint-deficient alleles of *rad3* may be unable to mediate the effect. *rad3*$^+$ encodes an extremely large protein (260 kD). Although the C-terminal kinase domain of *rad3*$^+$ is essential for function (Bentley *et al.*, 1996), it is not sufficient for Rad3p function (see Chapter 2). In addition, evidence suggests that protein-protein interaction sites may be located in the N-terminus of Rad3p (Chapter 2). In this series of experiments, it is important to note that the *cdc6-23* cells contain a wild-type copy of *rad3*$^+$ at the genomic locus. Here, we are investigating what elements of *rad3*$^+$ are required to mediate the overexpression phenotype.

To determine what sequences in *rad3*$^+$ are required for the synthetic dosage phenotype with *cdc6*, we assayed the ability of various *rad3* alleles to cause the cell cycle arrest when overexpressed in *cdc6-23* cells. *cdc6-23* cells overexpressing the *rad3-N775* N-terminal and the *rad3-C725* C-terminal fragments were grown on plates under inducing conditions for 3 days, and the phenotypes of the cells were examined. We have previously shown that overexpression of *rad3-N775* acts as a dominant negative and partially

compromises the S-M checkpoint of wild-type cells (Chapter 2). *rad3-C725*, which contains the Rad3p kinase domain, causes no new phenotypes when overexpressed (Chapter 2). In contrast to full-length, wild-type *rad3*$^+$, overexpression of these *rad3* deletion constructs did not result in the elongation of *cdc6-23* cells at the permissive temperature (Table 2). These data demonstrate the specificity of the interaction between *rad3*$^+$ and *cdc6*, since not all *rad3* alleles cause the synthetic phenotype. It should be noted that interpretation of the *rad3-N775* phenotype is complicated since overexpression of this allele partially compromises the S-M checkpoint in wild-type cells, and the elongation phenotype is dependent on an intact S-M checkpoint pathway (see below). However, overexpression of another allele, *rad3-LZ* , which harbors a twenty-two amino acid deletion of the putative N-terminal leucine zipper, was capable of causing the elongation phenotype (Table 2, and Figure 4C). This result is particularly interesting, since the *rad3-LZ* allele is non-functional with respect to checkpoint control; however, this allele does retain Rad3p kinase activity (Chapter 2). Thus, overexpression of checkpoint-deficient alleles of *rad3* can cause the synthetic dosage phenotype.

We also wished to determine if the synthetic dosage phenotype caused by overexpression of *rad3*$^+$ requires Rad3p kinase activity. The kinase domain of Rad3p is necessary, but not sufficient, for the protein's checkpoint function (Bentley *et al.*, 1996; Chapter 2). To determine if the synthetic phenotype requires kinase activity, we wanted to examine ability of *rad3-KD*, the kinase dead form of *rad3*$^+$, to cause elongation in *cdc6-23* cells. However, overexpression of *rad3-KD* not only partially compromises S-M checkpoint

Figure 4. Rad3p kinase activity is not required for the synthetic dosage phenotype with *cdc6-23*. Synthetic dosage assay was performed as described in Figure 2. Photomicrographs of *cdc6-23* (TE697) cells expressing rep3x vector control (pTE101) (A), rep1-*rad3*$^{+}$ (pTE157) (B), rep1-*rad3-LZ* (pTE716) (C), and *rad3-LZ-KD* (pTE743) (D) are shown.


A
B
vector
rad3+
C
D
rad3-LZ
rad3-LZ-KD

control, but it is also toxic to cells over many generations (Bentley *et al.*, 1996). To overcome this difficulty, we took advantage of the fact that mutation of the leucine zipper (LZ) greatly alleviates both the toxicity and dominant negative capabilities of the kinase dead allele (Chapter 2). In addition, the *rad3-LZ* allele retains the ability to cause the synthetic phenotype with *cdc6* (Table 2). Thus, we could determine whether the kinase domain was required for the synthetic interaction by investigating the phenotype of overexpression of the double mutant *rad3-LZ-KD* in *cdc6-23* cells. As shown in Figure 4D, overexpression of the *rad3-LZ-KD* allele causes elongation in *cdc6-23* alleles. A low number of aberrant mitoses or "cuts" forms under these conditions (approximately 8%), presumably because this construct still retains some dominant negative activity; "cuts" occur when cytokinesis and septation take place before DNA replication is completed, resulting in anucleate cells and cells in which the septum has bifurcated the nucleus (Enoch and Nurse, 1990). However, the majority of *cdc6-23* cells overexpressing *rad3-LZ-KD* exhibit a long, extended cell cycle arrest morphology. Thus, we conclude that kinase activity of Rad3p is not required for the synthetic interaction with *cdc6*.

Overexpression of *rad3*$^+$ interferes with DNA replication in *cdc6-23* cells.

There are a number of explanations for the synthetic dosage phenotype exhibited by *OPrad3*$^+$ *cdc6-23* cells. First, overexpression of *rad3*$^+$ could interfere with DNA replication in these particular mutants. Alternatively, overexpression of *rad3*$^+$ could cause DNA damage in these cells. A third possibility is that *rad3*$^+$ overexpression in these cells activates a "false"

checkpoint signal, amplifying the signal from a weak DNA replication defect, resulting in "unnecessary" cell cycle arrest. These models have different predictions, which are tested here.

If overexpression of *rad3*+ worsens the DNA replication defects or increases DNA damage in *cdc6-23* cells, it follows that these cells would have an increased requirement for an intact checkpoint pathway. Normally, *cdc6-23* cells are viable in combination with *cdc2-3w* or *chk1Δ* mutations at the permissive temperature of 25°C, but form "cuts" at 37°C (Iino and Yamamoto, 1997). The *cdc2-3w* mutation causes a disruption of the S-M checkpoint control in fission yeast (Enoch and Nurse, 1990), but cells with this mutation are largely proficient in the DNA damage checkpoint (Sheldrick and Carr, 1993). In contrast, *chk1Δ* mutants are mainly deficient in the DNA damage checkpoint (Walworth *et al.*, 1993), although *chk1*+ also contributes to the DNA replication checkpoint as well (Francesconi *et al.*, 1997; Boddy *et al.*, 1998; Lindsay *et al.*, 1998; Zeng *et al.*, 1998). If the DNA replication defects of *cdc6-23* cells overexpressing *rad3*+ are worse than *cdc6-23* cells expressing vector controls, then *cdc6-23 cdc2-3w* cells expressing *rad3*+ might form aberrant mitoses or "cuts" at the permissive temperature. However, if *rad3*+ causes DNA damage in *cdc6-23* cells, we might expect that *cdc6-23 chk1Δ* cells would form "cuts" at the permissive temperature. Alternatively, if *rad3*+ overexpression acts only to amplify the existing checkpoint signal in *cdc6-23* cells without increasing DNA structural problems associated with DNA replication arrest or DNA damage, we might expect *cdc2-3w* and *chk1* mutations to suppress the cell cycle arrest phenotype, since these genes act downstream of *rad3*+ in checkpoint control (see Figure 1). In other

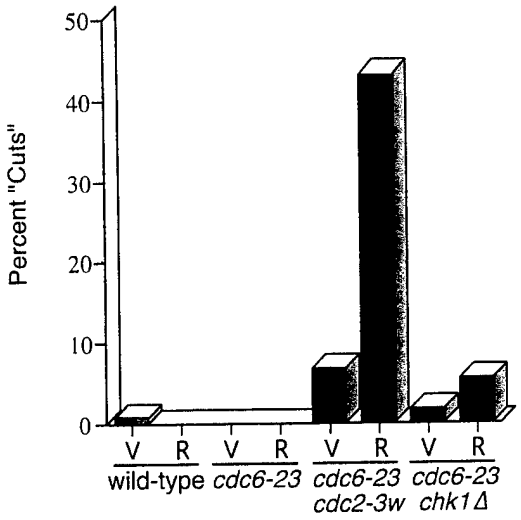
words, instead of elongating, *OPrad3+ cdc6-23 cdc2-3w* should divide normally and be indistinguishable from *cdc6-23 cdc2-3w* cells transformed with vector alone. The same would hold true for *cdc6-23 chk1Δ* double mutants.

To distinguish between these different models, rep3x vector controls and the rep1-*rad3+* plasmid were transformed into *cdc6-23 cdc2-3w* and *cdc6-23 chk1Δ* strains. Strains were inoculated into minimal media without thiamine to induce expression from the *nmt1+* promoter. Cells were fixed both at the point of inoculation into thiamine-minus media and at 45-48 hours afterwards. The cells were stained with DAPI, and the number of aberrant mitoses ("cuts") in each culture was counted (see Figure 5A). The length of the cells was also visually assessed. Photomicrographs of the cells are shown in Figure 5B. As shown, *cdc6-23 cdc2-3w* cells expressing *rad3+* exhibited at least a 10-fold increase in the percent of aberrant mitoses, or "cuts," compared with vector controls (see arrows in 5B). The appearance of "cuts" in the *cdc6-23 cdc2-3w* cells expressing *rad3+* indicates that these cells have an increased requirement for an intact S-M checkpoint pathway. These data strongly suggest that *rad3+* overexpression does not cause a "false" checkpoint signal, but actually interferes with DNA replication in these cells. In contrast, *cdc6-23 chk1Δ* cells expressing *rad3+* did not exhibit aberrant mitoses, suggesting that overexpression of *rad3+* overexpression does not cause significant DNA damage.

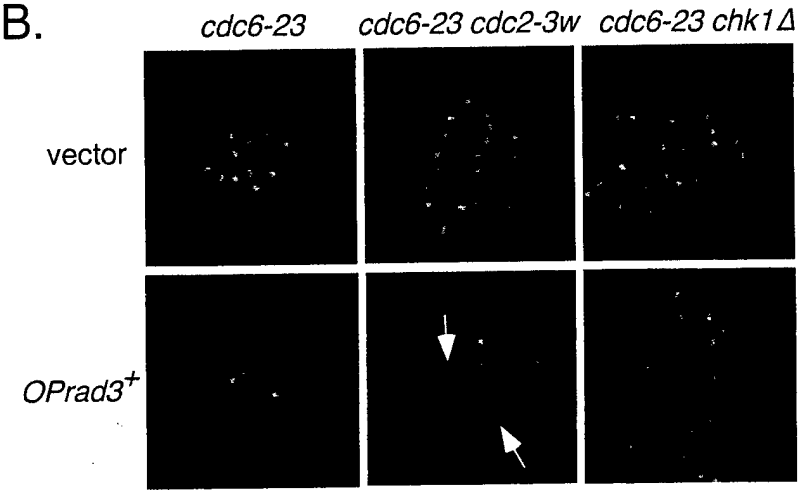
Although *OPrad3+ cdc6-23 chk1Δ* cells do not form "cuts," these cells also fail to elongate (compared to *cdc6-23* cells overexpressing *rad3+* ; see Figure 5B). As noted above, *chk1Δ* cells are mainly defective in the DNA damage checkpoint response, but recent data from a number of labs has revealed a role for

Figure 5. *OPrad3*$^+$ *cdc6-23* cells have an increased requirement for an intact S-M checkpoint pathway. (A) The percent aberrant mitoses ("cuts") exhibited by wild-type (TE235), *cdc6-23* (TE697), *cdc6-23 cdc2-3w* (TE860), and *cdc6-23 chk1Δ* (TE933) cells expressing vector (indicated by **V**) (pTE101) or rep1-*rad3*$^+$ (indicated by **R**) (pTE157) is indicated. Cells were grown under inducing conditions for 48 hours. "Cuts" form when cell division occurs before DNA replication is complete, resulting in anucleate cells or cells in which the septum has bifurcated the nucleus. Repeat experiments yielded similar results. (B) Pictures of cells from (A) grown under inducing conditions for 48 hours. Arrows point to examples of "cut" cells. (C) After 48 hours under inducing conditions, approximately 500 cells were plated back onto plates which repressed expression from the *nmt1*$^+$ promoter to assess viability. Colony growth was observed after 4 days of growth at 25˚C. Photographs are shown of plates containing *cdc6-23* cells expressing *rep1-rad3*$^+$, as well as *cdc6-23 chk1Δ* cells expressing vector and *rep1-rad3*$^+$.

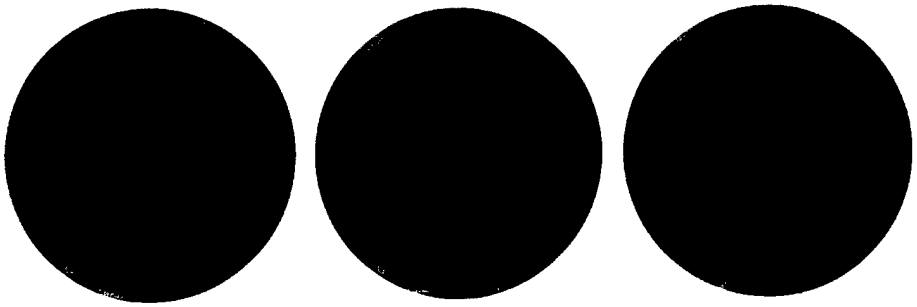
A.


Percent "Cuts"
50
40
30
20
10
0
V R V R V R V R
wild-type
cdc6-23
cdc6-23 cdc2-3w
cdc6-23 chk1Δ

B.


cdc6-23
cdc6-23 cdc2-3w
cdc6-23 chk1Δ
vector
OPrad3+

C.


cdc6-23 / OPrad3+
cdc6-23 chk1Δ / vector
cdc6-23 chk1Δ / OPrad3+

chk1+ in the DNA replication response as well (Zeng *et al.*, 1998; Francesconi *et al.*, 1997; Boddy *et al.*, 1998; Lindsay *et al.*, 1998). Thus, there are at least two possible explanations for the lack of elongation in *cdc6-23 chk1Δ* cells. The first is that *chk1*+ is required for *rad3*+ to interfere with DNA replication in *cdc6-23* cells. If the *chk1Δ* mutation suppresses the effects of *rad3*+ overexpression, it follows that the viability of *OPrad3*+ *cdc6-23 chk1Δ* cells should roughly match the viability of *cdc6-23 chk1Δ* cells expressing vector controls. Alternatively, the morphology of these cells could reflect a partial loss of the checkpoint pathway activation, such that the cells do not elongate, but do not form "cuts" either. In this case, the viability of *OPrad3*+ *cdc6-23 chk1Δ* cells should be lower than *cdc6-23 chk1Δ* cells expressing vector alone.

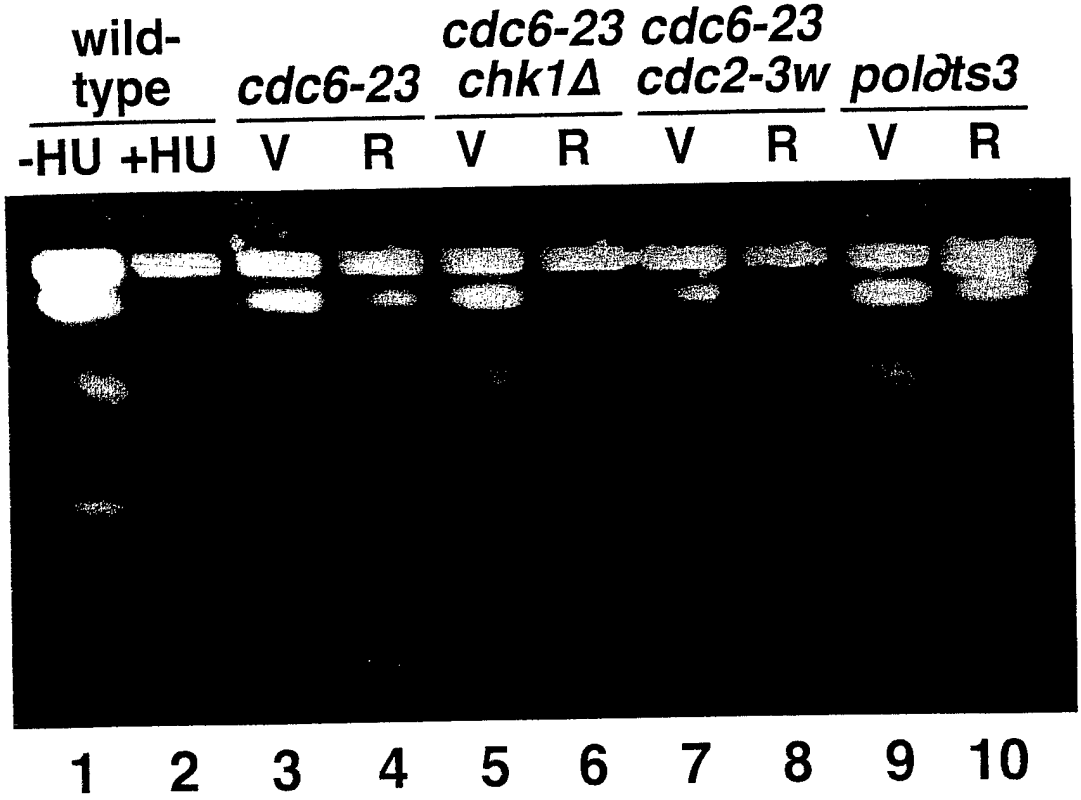
To distinguish between these alternatives, we examined the viability of *cdc6-23 chk1Δ* strains expressing *rad3*+ or vector controls. We grew cells for 48 hours under inducing conditions, then plated cells back onto plates containing thiamine to repress expression from the *nmt1*+ promoter; the ability of the cells to form colonies was examined after several days of growth. As shown in Figure 5C, *cdc6-23 chk1Δ* cells expressing *rad3*+ are less viable than *cdc6-23 chk1Δ* cells expressing vector alone. They are also less viable than *cdc6-23* cells expressing *rad3*+. We conclude that *chk1Δ* does not suppress the negative effects of *rad3*+ overexpression, but instead *chk1*+ contributes to the viability of the cells under these conditions. In summary, our data indicate that *rad3*+ overexpression is interfering with DNA replication in *cdc6-23* cells, since *OPrad3*+ *cdc6-23* cells have an increased requirement for an intact S-M checkpoint pathway.

Bulk DNA replication takes place in *cdc6-23* cells expressing *rad3*$^+$.

Thus far, our results suggest that overexpression of *rad3*$^+$ inhibits the ability of *cdc6-23* cells to complete DNA replication (Figure 5A and 5B). To examine the chromosome structure of these cells more directly, we used pulsed field gel electrophoresis, a technique which allows the separation of the three chromosomes of *S. pombe*. The ability or inability of chromosomes to be separated by pulsed field gel electrophoresis can be used to assess whether DNA replication has occurred to completion, possibly because unresolved replication intermediates, such as forks or bubbles in the DNA, prevent the DNA from migrating into the gel (Hennessy *et al.*, 1991). For example, chromosomes prepared from cells treated with hydroxyurea (HU), which inhibits DNA replication, do not migrate into the gel (e.g. Tournier *et al.*, 1996; Tanaka *et al.*, 1999).

To perform pulsed field gel electrophoresis, chromosomal DNA plugs were prepared from log-phase cells of different strains, grown in a variety of conditions. As shown in Figure 6, as expected, the chromosomes from exponentially growing wild-type cells were able to enter the gel (Lane 1), while the chromosomes from HU-treated wild-type cells were unable to do so (Lane 2). Cells for the remaining samples were grown in inducing conditions for the *nmt*$^+$ promoter for 48 hours. Chromosomes prepared from *cdc6-23* (Lane 3) and *pol∂ts3* (Lane 9) cells expressing rep3x vector controls enter the gel. The chromosomes of *OPrad3*$^+$ *cdc6-23* cells were slightly inhibited in their ability to enter the gel (Lane 4), compared to chromosomes from *cdc6-23* cells expressing vector controls (Lane

Figure 6. Pulsed field gel electrophoresis indicates that bulk DNA replication is taking place in *OPrad3*$^{+}$ *cdc6-23* cells. DNA prepared from approximately the same number of cells (as determined by counting cell number with a haeomocytometer) was loaded in each well. Wild-type cells (TE235) transformed with rep3x vector control grown in EMM (Lane 1), or in EMM containing 10 mM HU for 4 hours (Lane 2). Cells used to prepare the remaining samples were grown as described in Figure 2, and plugs were prepared after 48 hours of growth in thiamine-free medium. *cdc6-23* cells (TE697) expressing rep3x vector (pTE101) (Lane 3) or rep1-*rad3*$^{+}$ plasmid (Lane 4); *cdc6-23 chk1::ura4*$^{+}$ cells (TE933) expressing rep3x vector (Lane 5) or *rad3*$^{+}$ (Lane 6); *cdc6-23 cdc2-3w* cells (TE860) expressing rep3x vector (Lane 7) and *rad3*$^{+}$ (Lane 8); *pol∂ts3* cells expressing rep3x vector (Lane 9) or *rad3*$^{+}$ (Lane 10).


wild-type
cdc6-23
cdc6-23 chk1Δ
cdc6-23 cdc2-3w
polδts3
-HU +HU
V R V R V R V R
1 2 3 4 5 6 7 8 9 10

3). However, overexpression of *rad3*$^+$ also seemed to inhibit separation of the *pol∂ts3* chromosomes as well, indicating that the subtle effects of *rad3*$^+$ overexpression on chromosome separation may not be specific to the *cdc6-23* cells (Lane 10). *pol∂ts3* cells are not particularly sensitive to *rad3*$^+$ overexpression. From these data, we conclude that *rad3*$^+$ overexpression does not completely prevent DNA replication in these strains. In agreement with this result, FACS analysis showed that the vast majority of *cdc6-23* cells overexpressing *rad3*$^+$ have a G2 DNA content (data not shown), confirming that bulk DNA replication has occurred.

The chromosomes of *cdc6-23 chk1Δ* mutants and *cdc6-23 cdc2-3w* mutants expressing vector controls were also capable of being separated by pulsed field electrophoresis (Lanes 5 and 7, respectively), indicating that bulk DNA replication also takes place in these double mutants. Overexpression of *rad3*$^+$ in *cdc6-23 chk1Δ* and *cdc6-23 cdc2-3w* strains greatly decreased the ability of the chromosomes to be separated by pulsed field electrophoresis, compared with the chromosomes from the same strains expressing vector alone (compare lane 6 with lane 5; and lane 8 with lane 7) and from *cdc6-23* cells expressing *rad3*$^+$ (Lane 4). Strikingly, most of the chromosomes of *OPrad3*$^+$ *cdc6-23 cdc2-3w* cells fail to enter the gel (Lane 8). However, a smear of DNA is apparent towards the bottom of the gel, suggesting that chromosomal breaks may be occurring, as cells are proceeding into mitosis with damaged or unreplicated DNA. Indeed, *OPrad3*$^+$ *cdc6-23 cdc2-3w* exhibit aberrant mitoses in these conditions. The pulsed field gel electrophoresis data suggest that the cell elongation and cell cycle delay exhibited by *cdc6-23* cells overexpressing *rad3*$^+$

is needed to finish DNA replication and/or repair. This interpretation agrees with our data which showed that an checkpoint pathway is required for the viability of *OPrad3+cdc6-23* cells (Figure 5). Taken together, our data suggest that *rad3+* overexpression interferes with DNA replication in *cdc6-23* cells, but does not prevent bulk DNA replication from taking place. *chk1+* and *cdc2+* are required to delay cell cycle progression under these circumstances, which presumably allows time for the cells to complete the last steps of DNA replication and/or repair.

Synthetic lethality between *rad3* and *cdc6* is also allele dependent.

We have demonstrated an allele-specific genetic interaction between overexpressed *rad3+* and *cdc6*. Although we did not use synthetic lethality as an initial screen to identify genetic interactions between *rad3* and DNA replication proteins, double mutant analysis can provide valuable insights. Therefore, we investigated how a loss of *rad3* function affects the different *cdc6* alleles.

To investigate the phenotype of *rad3Δ cdc6* double mutants, we performed tetrad analysis on crosses between *rad3Δ* strains and each of five strains carrying different *cdc6* alleles (see Table 3). *rad3Δ polδts2* mutants were easily recovered, indicating that *rad3* and the *polδts2* allele are not synthetically lethal (see Table 3A). We were also able to recover *rad3Δ cdc6-121* mutants, as well as *rad3Δ polδts3* mutants, although these double mutants were extremely sick and not all of these spores were capable of forming colonies (see Table 3B and C). Indeed, another group reported that *rad3Δ* is synthetically lethal with

Table 3. Synthetic lethality studies between *rad3Δ* and *cdc6*.

A. *rad3Δ x polδts2* (TE981 x TE985)
Total: 74/88 spores (84% viability)

genotype	number of spores	expected
wild-type	20	22
rad3Δ	17	22
polδts2	19	22
rad3Δ polδts2	18	22

B. *rad3Δ x cdc6-121* (TE988 x TE982)
Total: 55/84 spores (65% viability)

genotype	number of spores	expected
wild-type	16	21
rad3Δ	16	21
cdc6-121	16	21
rad3Δ cdc6-121	5	21

C. *rad3Δ x polδts3* (TE988 x TE986)
Total: 91/120 spores (75% viability)

genotype	number of spores	expected
wild-type	27	30
rad3Δ	31	30
polδts3	29	30
rad3Δ polδts3	3	30

D. *rad3Δ x cdc6-23* (TE981 x TE984)
Total: 60/96 spores (62.5% viability)

genotype	number of spores	expected
wild-type	16	24
rad3Δ	23	24
cdc6-23	21	24
rad3Δ cdc6-23	0	24

E. *rad3Δ x polδts1* (TE988 x TE987)
Total: 50/84 spores (60% viability)

genotype	number of spores	expected
wild-type	17	21
rad3Δ	12	21
polδts1	21	21
rad3Δ polδts2	0	21

F. *hus1Δ x cdc6-23* (TE482 x TE908)
Total: 56/84 (66.6% viability)

genotype	number of spores	expected
wild-type	18	21
hus1Δ	19	21
cdc6-23	19	21
hus1Δ cdc6-23	0	21

pol∂ts3 (Uchiyama *et al.*, 1997). Interestingly, we were not able to obtain any double mutants in the crosses between *rad3Δ* and *cdc6-23* or *pol∂ts1,* the two *cdc6* alleles which interact with *OPrad3*$^+$ (Table 3D and E). These data further support our conclusion that there is an allele-dependent interaction between *rad3* and *cdc6*.

In order to investigate whether the synthetic lethality between *rad3* and *cdc6-23* was specific to *rad3,* tetrad analysis was performed on the asci resulting from a cross between another "*checkpoint rad*" mutant, *hus1Δ*, and *cdc6-23*. We were unable to recover any *hus1Δ cdc6-23* double mutants from this cross, indicating that *cdc6-23* exhibits synthetic lethality with *hus1Δ* (see Table 3F). This indicates that the synthetic lethality of *rad3* with *cdc6-23* may be due to a lack of checkpoint control, rather than to a specific interaction between *rad3* and *cdc6*. In contrast, using tetrad analysis, we were able to isolate *cdc6-23 chk1Δ, cdc6-23 cdc2-3w,* and *cdc6-23 cds1Δ* mutants (see Figure 5 and Table 6) , in agreement with the results of others (Uchiyama *et al.*, 1997; Iino and Yamamoto, 1997). *chk1*$^+$, *cds1*$^+$, and *cdc2*$^+$ are all involved in the fission yeast checkpoint pathway (see Figure 1). As reported by others, *cdc6-23 chk1Δ* and *cdc6-23 cdc2-3w* cells both "cut" at the restrictive temperature of 37°C, which indicates that *chk1*$^+$ and *cdc2*$^+$ are required for the cell cycle arrest exhibited by *cdc6-23* cells at 37°C (data not shown)(Iino and Yamamoto, 1997). In contrast to the synthetic lethality between *cdc6-23* and *hus1Δ* or *rad3Δ* mutations, it is likely that the *cdc2-3w*, *chk1*Δ, and *cds1Δ* double mutants are viable at 25°C because these mutations do not completely abolish all aspects of the S-M checkpoint response.

***pol∂ts1* and *cdc6-23* mutants exhibit low nonpermissive temperatures.**

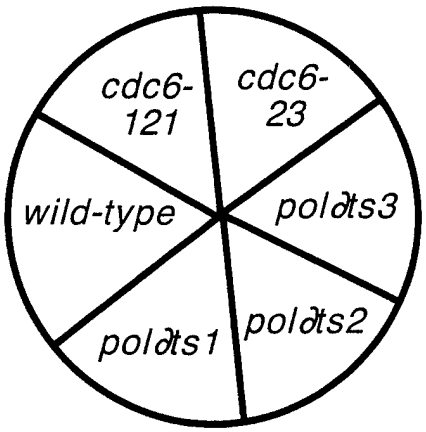
pol∂ts1 and *cdc6-23* mutants exhibit the synthetic dosage phenotype with *rad3*$^+$, while the other *cdc6* alleles do not. In addition, these alleles exhibit synthetic lethality with loss of *checkpoint rad* gene function, while the other alleles are capable of surviving without an intact S-M checkpoint pathway (see Table 3). The sites of mutation of four of the five alleles have been determined (see Table 4). Interestingly, the mutations in the two alleles which show the phenotype are both located in the N-terminus of the protein. Thus, it is possible that the *cdc6-23* and *pol∂ts1* mutants have similar molecular defects, and that the synthetic dosage interaction caused by *rad3*$^+$ overexpression is related to the particular *cdc6*$^+$ function affected by these N-terminal mutations. To further our understanding of the differences between the *cdc6* alleles, we investigated whether *pol∂ts1* and *cdc6-23* exhibit quantitatively different growth defects from the other *cdc6* alleles.

To test the growth of strains carrying the different *cdc6* alleles, *cdc6-23*, *cdc6-121*, *pol∂ts1*, *pol∂ts2*, *pol∂ts3*, and wild-type strains were streaked onto YE5S (rich medium) plates and these plates were placed at 25°C, 29°C, and 32°C; growth of the cells was observed after two days. As shown in Figure 7, the *cdc6-23* and *pol∂ts1* strains exhibited lower nonpermissive temperatures than the other *cdc6* alleles. Although *cdc6-23* and *pol∂ts1* mutants exhibit only slight defects at 25°C, the cells are clearly less able to form colonies at 29°C and 32°C. The other alleles appear to have higher nonpermissive temperatures, as they are capable of growth at 29°C. Similar results were obtained using minimal plates (data not shown). In agreement with our results, Liu *et al.* recently reported that the

Table 4. Molecular mutations in different *cdc6* (pol∂) alleles.

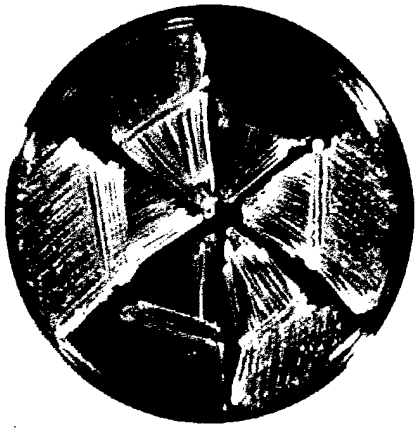
Allele	Mutation/Reference
cdc6-23	E227K (Iino and Yamamoto, 1997)
pol∂ts1	A143V, P144S (Francesconi *et al.*, 1993)
pol∂ts2	E271K
pol∂ts3	R1064Q
cdc6-121	N.D.

Figure 7. *pol∂ts1* and *cdc6-23* have lower nonpermissive temperatures than the other *cdc6* alleles examined. Cells were streaked onto YE5S (rich medium) plates, and grown for 2 days at the indicated temperatures. Strains were wild-type (TE235), *pol∂ts1* (TE908), *pol∂ts2* (TE907), *pol∂ts3* (TE905), *cdc6-23* (TE697), and *cdc6-121* (TE754). Streaks on EMM5S (minimal medium) yielded similar results (data not shown).

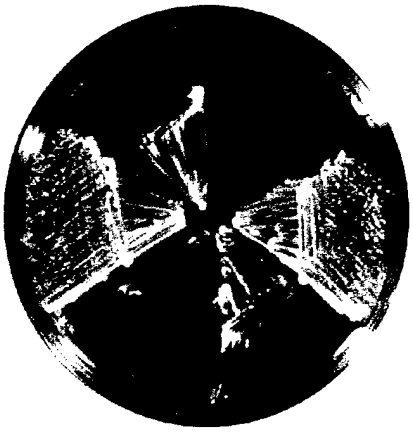


Key

25°C



29°C



32°C

permissive temperatures for *cdc6-23* and *pol∂ts1* are among the lowest of a group of DNA replication mutants, which included *cdc1-7*, *cdc20-M10*, and *cdc19-P1* (Liu *et al.*, 1999). However, in contrast to our findings, this group determined that *cdc6-23*, *pol∂ts1*, and *pol∂ts2* all have similar growth defects. This may be because they used different methods to assess the permissive temperatures of the strains (Liu *et al.*, 1999). Interestingly, while we find many similarities between the *cdc6-23* and *pol∂ts1* alleles, this group found that *pol∂ts1* cells exhibits increased rates of deletion of sequences (a mutator phenotype), while *cdc6-23* cells do not (Liu *et al.*, 1999). Thus, the mutator phenotype displayed by certain *cdc6* alleles appears to be separable from the synthetic dosage phenotype between *rad3*$^+$ and *cdc6*.

Overexpression of *rad3*$^+$ causes a *chk1*$^+$-dependent extension of the S phase block in HU.

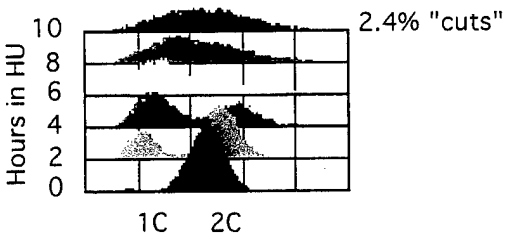
Our data suggest that overexpression of *rad3*$^+$ has a detrimental effect on DNA replication in specific *cdc6* mutants. Although *rad3*$^+$ did not show a synthetic dosage interaction with other DNA replication mutants, we wished to investigate whether overexpression of *rad3*$^+$ had noticeable effects on cells when DNA replication is compromised by hydroxyurea (HU), which inhibits ribonucleotide reductase. We investigated whether overexpression of *rad3*$^+$ causes hydroxyurea (HU)-treated wild-type cells to elongate more than vector controls. We found that wild-type cells overexpressing *rad3*$^+$ are not longer than rep3x controls in HU (data not shown). In addition, overexpression of

rad3$^+$ does not cause a significant decrease in the viability of wild-type cells treated with HU (see Chapter 2).

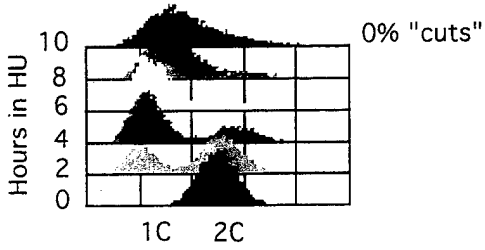
However, using fluorescence activated cell sorting (FACS) analysis, we noticed that overexpression of *rad3*$^+$ in wild-type cells can cause an extension of the S phase block during a 10 hour HU exposure. Using FACS analysis, the DNA content of a population of cells can be measured. As shown in Figure 8, most fission yeast cells have a 2C DNA content prior to exposure to HU, since the G2 phase comprises approximately 70% of the cell cycle. In wild-type cells expressing vector controls (Figure 8A), a 1C DNA peak is evident after only a 2 hour exposure to HU. By 4-6 hours in HU, the majority of cells have a 1C DNA content, indicating that an S phase block has occurred. After 6 hours in HU, wild-type cells expressing vector controls begin to synthesize DNA inefficiently, slowly escaping from the HU arrest. This phenomenon has been observed by others (Sazer and Nurse, 1994). Although overexpression of *rad3*$^+$ does not prevent the S phase block from occurring in wild-type cells exposed to HU, overexpression of *rad3*$^+$ markedly represses DNA synthesis in the presence of HU, such that even after 10 hours, the majority of the cells have a 1C DNA content (Figure 8B). Overexpression of the *rad3-KD* allele was also capable of causing an extended S phase block in wild-type cells (see Table 5)(Chapter 2), indicating that the kinase activity of Rad3p is not required for this effect. However, the *rad3-N775* allele was incapable of causing the extended S phase arrest in HU (Table 5)(Chapter 2). Thus, there are similarities between the ability of *rad3*$^+$ to extend S phase in HU and to cause the synthetic dosage phenotype

Figure 8. Overexpression of *rad3*+ causes a *chk1*+-dependent extension of the S phase block in HU. FACS analysis of cells incubated with 10 mM HU for indicated times (at left). To prepare cells for FACS, cells were fixed with 70% ethanol and stained with propidium iodide. Wild-type cells (TE235 or TE239) expressing rep3x vector control (pTE101) (A), rep1-*rad3*+ (pTE157) (B) and rep1-*rad1*+ (pTE567) (C) plasmids are shown, as well as *rad1Δ* cells (TE459) (D) and *chk1Δ* cells (TE255) (E) expressing rep1-*rad3*+. FACS profiles of *rad1Δ* cells and *chk1Δ* cells expressing vector controls were similar to those of wild-type cells expressing vector controls (data not shown). The percentage of cells undergoing aberrant mitoses ("cuts") in each culture after 10 hours incubation in HU is indicated (at right). The percentage shown is the average of results from two independent experiments.

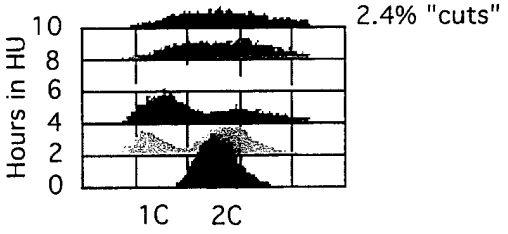
A wild-type/vector



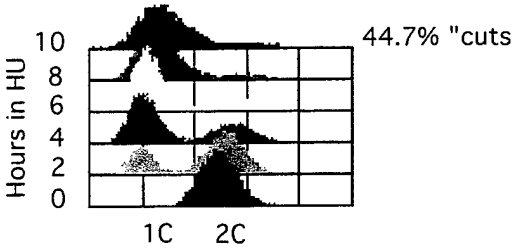
B wild-type/*OPrad3*$^{+}$



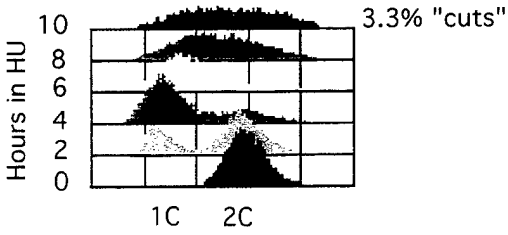
C wild-type/*OPrad1*$^{+}$



D *rad1Δ/OPrad3*$^{+}$



E *chk1Δ/OPrad3*$^{+}$



in *cdc6-23* cells. In particular, neither of the overexpression phenotypes requires Rad3p kinase activity.

To further our understanding of the ability of *rad3*$^+$ overexpression to cause an extended S phase in HU, we investigated this phenotype further. First, we were interested to see if the extended S phase arrest was specific to overexpression of *rad3*$^+$. To determine if overexpression of another *checkpoint rad* gene, *rad1*$^+$, also inhibits the recovery of DNA synthesis, we examined the FACS profiles of wild-type cells expressing rep1-*rad1*$^+$ during a 10 hour HU exposure. As shown in Figure 8C, these cells exhibited FACS profiles which were very similar to those of wild-type cells expressing vector alone (Figure 8A). Wild-type cells overexpressing *rad1*$^+$ started to synthesize DNA as early as 6 hours after HU addition. From these data, we conclude that the ability of *rad3*$^+$ overexpression to extend S phase arrest in HU is at least somewhat specific, since another *checkpoint rad* gene, *rad1*$^+$, is incapable of causing this phenotype.

We were also interested in determining what other gene products are required for the extended S phase arrest in HU. To do this, we examined the FACS profiles of *rad1Δ* and *chk1Δ* cells expressing the rep3x vector control or rep1-*rad3*$^+$ over a 10 hour HU exposure. *rad1Δ* and *chk1Δ* cells expressing rep3x vector controls mimicked the FACS profiles of wild-type cells expressing vector controls (data not shown). Overexpression of *rad3*$^+$ in *rad1Δ* cells caused an extension of S phase arrest in HU, as the cells maintained a 1C peak for up to 10 hours in HU (Figure 8D). From this, we conclude that *rad1*$^+$ is not required for *rad3*$^+$-mediated inhibition of DNA synthesis in this assay. In striking contrast, *chk1Δ* cells overexpressing *rad3*$^+$ did not maintain the S phase block

(Figure 8E). By 8 hours the cells are clearly escaping from HU arrest, since the peak is drifting towards a 2C DNA content. These data indicate that the extension of S phase in HU caused by overexpression of *rad3*$^+$ is dependent on *chk1*$^+$. Thus, *chk1*$^+$ may have a role in regulating DNA replication. This is a remarkable result, as previous studies have suggested that *chk1*$^+$ mainly responds to DNA damage.

In order to assess whether different strains arrested cell division in hydroxyurea, cells that had been incubated in hydroxyurea for 0 and 10 hours were stained with the DNA-dye DAPI and examined by fluorescence microscopy. The length of the cells was observed, and the percentage of aberrant mitoses in each culture was calculated (see Figure 8). *rad1Δ* cells expressing either vector controls or rep1-*rad3*$^+$ failed to delay mitosis, as 48.5% and 44.7% of the cells undergo aberrant mitoses after 10 hours incubation in HU, respectively. However, wild-type cells and *chk1Δ* cells expressing vector or *rad3*$^+$ all retained the mitotic checkpoint, since in each of these strains, most cells elongated and less than 5% exhibited aberrant mitoses after 10 hours incubation in HU (see Figure 8 and data not shown). Therefore, our data clearly demonstrate that the extension of S phase arrest caused by *rad3*$^+$ overexpression is not dependent on mitotic checkpoint arrest in HU (see Table 5). Wild-type cells expressing *rad3*$^+$ have an intact mitotic checkpoint and display the extended S phase arrest, while *rad1Δ* cells expressing vector controls lack both the checkpoint and the G1 maintenance. In these examples, the S phase arrest and mitotic arrest occur together. However, *rad1Δ* cells expressing *rad3*$^+$ and wild-type cells expressing *rad3-KD* are both checkpoint deficient but both of these strains clearly exhibit

Table 5. Extension of S phase arrest in HU is separable from mitotic delay.

Strain	Mitotic Checkpoint	Extended S phase arrest
wild-type/ vector	+	-
wild-type/ *OPrad3*$^{+}$	+	+
wild-type/ *OPrad3-KD*	-	+
wild-type/ OPrad3-N775	-	-
rad1Δ/ vector	-	-
rad1Δ/ OPrad3^{+}	-	+
chk1Δ/ vector	+	-
chk1Δ/ OPrad3^{+}	+	-

extended S phase arrest, as the G1 peak is maintained for up to 10 hours in HU (Figure 8 and Chapter 2). In addition, *chk1Δ* cells overexpressing *rad3*$^+$ arrest the cell cycle in HU, but fail to show the extended S phase arrest. Thus, we conclude that the extended S phase arrest in HU caused by overexpression of *rad3*$^+$ is distinct and separable from maintenance of the S-M checkpoint.

Discussion

In this study, we set out to identify interactions between the *S. pombe checkpoint rad* gene *rad3*$^+$ and DNA replication genes. We are interested in these interactions because DNA replication proteins are candidates to be both activators and targets of the *rad3*$^+$ checkpoint pathway. Here, we describe two conditions in which *rad3*$^+$ interferes with DNA replication, providing evidence that links *rad3*$^+$ to DNA replication.

rad3$^+$ interferes with DNA replication in *cdc6* cells

First, we describe our discovery of a specific synthetic dosage interaction between *rad3*$^+$ and *cdc6*, which encodes pol∂. Overexpressing *rad3*$^+$ in two *cdc6* mutants, *cdc6-23* and *pol∂ts1*, causes the cells to elongate at 25°C, the permissive temperature. *rad3*$^+$ does not cause this phenotype in the other alleles of *cdc6*, or in other DNA replication mutants we tested (see Figures 2 and 3). In addition, overexpression of other checkpoint genes does not specifically cause this phenotype in *cdc6* cells (Table 2). Thus, the interaction between *rad3*$^+$ and *cdc6* appears to be specific.

Since mutations in *cdc6* may cause defects in DNA replication even at the permissive temperature, one possibility is that *rad3*$^+$ worsens the *cdc6* phenotype by interfering with checkpoint function. However, we do not believe that *rad3*$^+$ is obstructing checkpoint function in these cells, since the elongation resembles the classic checkpoint-dependent cell division cycle (*cdc*) phenotype of many mutants which lack a function required for cell cycle progression. In addition, *OPrad3*$^+$ does not interfere with checkpoint function in wild-type cells (Chapter 2). We considered two other explanations for why *OPrad3*$^+$ *cdc6* cells arrest at the permissive temperature. First, *rad3*$^+$ overproduction might inhibit the ability of these mutants to perform DNA replication or DNA repair. Alternatively, *rad3*$^+$ overexpression might amplify an existing checkpoint signal.

We believe that *rad3*$^+$ overproduction is interfering with DNA replication in the *cdc6* mutants. In strong support of this, *OPrad3*$^+$ *cdc6-23* cells are hypersensitive to defects in the S-M checkpoint pathway (Figure 5). The S-M checkpoint pathway in fission yeast is only required when DNA replication is compromised, such as in situations where nucleotides are limiting (grown in the presence of the drug hydroxyurea), or when DNA processing enzymes are mutated. *cdc2-3w* mutants have a defective S-M checkpoint (Enoch and Nurse, 1990), but they are relatively radiation resistant (Sheldrick and Carr, 1993). *OPrad3*$^+$ *cdc6-23* cells have an increased requirement for S-M checkpoint control, since *OPrad3*$^+$*cdc6-23 cdc2-3w* cells undergo aberrant mitoses at the permissive temperature (Figure 5). In addition, *OPrad3*$^+$ *cdc6-23 chk1Δ* cells are less viable than *cdc6-23 chk1Δ* mutants expressing vector alone (Figure 5). This

increased demand for a functional checkpoint pathway indicates malfunctional DNA replication or repair.

We tried to obtain direct evidence for DNA replication defects in *OPrad3*$^+$ *cdc6-23* cells using pulsed field gel electrophoresis (Figure 7). Surprisingly, pulsed field gel electrophoresis indicates that bulk replication has taken place in these cells. However, the chromosomes from *cdc6-23 cdc2-3w* cells overexpressing *rad3*$^+$ are either unable to enter the gel or form a smear at the bottom of the gel. We presume that in this genetic background, the cells do not have enough time to complete DNA replication and repair before cell division takes place. Interestingly, FACS analysis has demonstrated that *cdc6-23* mutants, like some other DNA replication mutants (e.g. MacNeill *et al.*, 1996), arrest with an apparent G2 DNA content at their nonpermissive temperature (Iino and Yamamoto, 1997). Although lethal, these mutants may only be defective in replicating a small subset of the DNA. Therefore, it is possible that overexpression of *rad3*$^+$ causes a phenotype similar to that of *cdc6-23* cells grown at the restrictive temperature.

The alternative model, that *rad3*$^+$ amplifies an existing checkpoint signal, does not appear to be correct for two reasons. First, this model predicts that the elongation of *OPrad3*$^+$ *cdc6-23* cells is unnecessary, and that mutations in downstream components of the S-M checkpoint would restore normal cell division by suppressing the amplified signal. However, the data reviewed above indicate that the checkpoint-dependent cell cycle delay increases the ability of the cells to survive the effects of *rad3*$^+$ overexpression. In addition, this model predicts that checkpoint-deficient *rad3* alleles would be unable to cause the

elongation in *cdc6* cells. However, this prediction also does not hold true, since the checkpoint-defective *rad3-LZ* and *rad3-LZ-KD* alleles show the synthetic interaction with *cdc6* (Figure 4). Thus, we conclude that *rad3*+ overexpression adversely affects the ability of the *cdc6-23* cells to properly complete DNA replication.

How does *rad3*+ interfere with DNA replication in *cdc6-23* cells?

The ability of *rad3*+ overexpression to interfere with DNA replication in *cdc6-23* cells does not require *rad3*+ mitotic checkpoint function, since two checkpoint-defective alleles (*rad3-LZ* and *rad3-LZ-KD*) still mediate the effect. Particularly interesting is the fact that the catalytically inactive *rad3-LZ-KD* allele is able to cause the synthetic interaction with *cdc6*. This result indicates that kinase activity is not required for the phenotype, and further suggests that *rad3*+ is not functioning by increasing the phosphorylation of downstream substrates.

In order to understand the mechanism behind the synthetic interaction between *rad3*+ and *cdc6*, it is worth considering how the *cdc6-23* and *pol∂ts1* differ from the other *cdc6* alleles and DNA replication mutants tested. These two mutants, which both exhibit the synthetic phenotype with *rad3*+, have properties which distinguish them from the other *cdc6* alleles. Importantly, they appear to be quantitatively more severe than the other *cdc6* alleles tested, either in the amount or type of DNA replication problems they accrue. In support of this, *cdc6-23* and *pol∂ts1* have lower permissive temperatures than the other *cdc6* alleles tested (Figure 7). Our data showing that these two alleles are synthetically lethal with the *checkpoint rad* genes *rad3* and *hus1*, while the other three alleles

are not (Table 3), provides further evidence that these alleles may have qualitatively or quantitatively different defects than the other *cdc6* alleles. In addition, Liu *et al.* recently showed that the permissive temperatures for *cdc6-23* and *pol∂ts1* are among the lowest of a group of DNA replication mutants, which included *cdc1-7*, *cdc20-M10*, and *cdc19-P1* (Liu *et al.*, 1999). *cdc6-23* and *pol∂ts1* cells may have unique defects, or produce quantitatively more defects, that make them particularly sensitive to the effects of *rad3*$^+$ overexpression.

cdc6-23 and *pol∂ts1* both have molecular mutations which are located in the N-terminus of the protein (see Table 4). The mutations do not disrupt the 3'-5' exonuclease domain or the region required for dNTP/pyrophosphate binding, necessary for polymerase activity (defined in Zhang *et al.*, 1991). The region affected by these alleles may mediate an interaction with another subunit or DNA replication factor; in fact, one group found that the N-terminus of human pol∂ mediated its interaction with PCNA (Zhang *et al.*, 1995a; Zhang *et al.*, 1995b). However, pol∂ and PCNA may not directly interact (Tratner *et al.*, 1997; Zhou *et al.*, 1997). Thus, it is still unclear what function the N-terminus of pol∂ fulfills.

The altered gene dosage of *rad3*$^+$ may perturb the normal regulation of its interactions with other proteins. Possibly the synthetic phenotype caused by *OPrad3*$^+$ is the result of a titration effect; for example, the high levels of Rad3p may lower the activity of another protein in the cell by competing it away from its normal complexes. Allele-dependent interactions between genes sometimes indicate a direct interaction between the proteins the genes encode. We have considered the possibility of a direct interaction between Rad3p and Cdc6p. To investigate this, we performed coimmunoprecipitation assays using HA-tagged Cdc6p and myc-Rad3p (data not shown). Although HA-Cdc6p did

coimmunoprecipitate with myc-Rad3p, the binding observed was only slightly above background levels. In addition, we were unable to bring down myc-Rad3p when we immunoprecipitated HA-Cdc6p. Thus, we were unable to obtain unambiguous results supporting a strong interaction between these two proteins. However, it is possible that the interaction is transient, or that we are not looking at the interaction in biologically appropriate conditions.

rad3$^+$ overexpression may worsen the DNA replication defects in *cdc6-23* cells in a more indirect manner, such as titration of a protein which interacts with both Rad3p and pol∂. Here, the allele-specificity may be explained if the mutations in pol∂ reduce its ability to compete with Rad3p for the common factor. In yeast systems, pol∂ has been shown to genetically interact with subunits of RFC, RPA, and ORC (Xie *et al.*, 1999; Longhese *et al.*, 1994; Kroll *et al.*, 1996, Noskov *et al.*, 1998). Physical interactions have been demonstrated between pol∂ and Cdc1p, a subunit of pol∂ (MacNeill *et al.*, 1996), RFC (Hughes et al., 1999) and as mentioned above, pol∂ is likely to interact with PCNA. It is possible that Rad3p competes with pol∂ for interacting with any one of these factors. Since PCNA has been shown to function in cell cycle control in other contexts, we investigated the possibility that Rad3p binds to PCNA, the processivity factor for pol∂. Again, we were unable to conclusively demonstrate that Rad3p and PCNA interact, although certain assay conditions allowed us to see an interaction between these proteins (see Appendix 2). It will be easier to make firm conclusions regarding protein-protein interactions once better tools for following the Rad3p protein become available.

An additional replication phenotype conferred by *rad3*$^+$ overexpression.

In addition to interfering with DNA replication in specific *cdc6* alleles, *rad3*$^+$ overexpression also prolongs the S phase arrest of wild-type cells exposed to HU. This phenotype is intriguing for a number of reasons. First, the prolonged S phase arrest is not dependent on kinase activity of *rad3*$^+$, since overexpression of *rad3-KD* also maintains the G1 peak for up to 10 hours in HU (Chapter 2 and Table 5). The phenotype is also independent of mitotic arrest, since extension of S phase can occur in checkpoint-deficient and checkpoint-proficient cells (see Table 5). Additionally, prolonged S phase arrest appears to be *rad3*$^+$-specific, since overexpression of another *checkpoint rad* gene, *rad1*$^+$, is incapable of causing the extended S phase arrest. Finally, *rad3*$^+$ overexpression seems to exert its effects on S phase through *chk1*$^+$, since overexpression of *rad3*$^+$ does not cause the extension of S phase in *chk1Δ* mutants. This is a remarkable result, since it implies that *chk1*$^+$ may have a role in regulating S phase progression. Interestingly, *chk1*$^+$ has recently been shown to have a role in recovery from the late stages of HU-induced S phase arrest (Brondello *et al.*, 1999). The discovery that *rad3*$^+$ overexpression can inhibit resumption of DNA replication in wild-type cells recovering from an S phase arrest also demonstrates that the ability of *rad3*$^+$ overexpression to influence DNA replication is not confined to specific genetic backgrounds.

Models for *rad3*$^+$ overexpression phenotypes.

Although it is not entirely clear whether the ability of *OPrad3*$^+$ to extend

S phase in HU is related to its synthetic dosage interaction with *cdc6-23*, enough similarities between the two conditions exist to merit their comparison. Both situations involve overexpression of *rad3*$^+$ in conditions where DNA replication may be at least partially compromised. In both cases, the overexpression phenotype does not depend on *rad3*$^+$ kinase activity. *rad3-KD* is capable of causing the extension of S phase arrest in HU; *rad3-LZ-KD* alleles retain the ability to cause elongation of *cdc6-23* mutants. In addition, *rad3-N775* is unable to cause the synthetic dosage phenotype, or the extended G1 block in HU. However, there are also differences between the phenotypes. Particularly, the *cdc6* synthetic dosage phenotype appears to occur in late S or G2, while *rad3*$^+$ overexpression in HU seems to affect progression through early S phase. Although they could be coincidental, the strong correlations between these two *rad3*$^+$ overexpression phenotypes lead us to believe that they may be related. Below, we consider models which attempt to reconcile the findings from the two overexpression phenotypes.

It is intriguing that *rad3*$^+$ overexpression only appears to have detectable effects on DNA replication when the DNA polymerase may already be having difficulty functioning, such as in *cdc6-23* cells or in recovery from HU arrest. This suggests that *rad3*$^+$ overexpression may be adversely affecting recovery from stalled DNA replication. For example, overexpression of *rad3*$^+$ may titrate out a factor that helps cells respond to stalled replication forks. Cds1p is believed to have a role in recovery from S phase arrest. Indeed, *cdc6-23 cds1Δ* cells appear slightly elongated at 25°C. However, it seems unlikely that overexpression of *rad3*$^+$ functions simply by titrating away Cds1p, since *OPrad3*$^+$ has a

deleterious effect in *cdc6-23 cds1* cells. Specifically, *OPrad3*$^+$ causes *cdc6-23 cds1Δ* cells to exhibit aberrant mitoses at the permissive temperature (data not shown). Additionally, *cds1Δ* cells do not display an extended S phase arrest upon recovery from HU (data not shown). This indicates that inactivation of *cds1* activity does not mimic the phenotype of *OPrad3*$^+$, suggesting that *cds1*$^+$ cannot be the only target of *OPrad3*$^+$.

A second possibility is that overexpression of *rad3*$^+$ blocks the firing of late DNA replication origins in these conditions. *MEC1*, the budding yeast *rad3*$^+$ homologue, acts to delay the firing of late-firing origins in the presence of HU (Santocanale and Diffley, 1998). The mechanism behind this inhibition is not yet known, but one possibility is that *MEC1* phosphorylates downstream targets to exert its effects. *mec1Δ* mutants, which are normally inviable, are suppressed by extragenic mutations in the DNA replication genes *CDC7* and *DBF4*, which are both involved in ORC firing (Desany *et al.*, 1998). Since mutations which increase the availability of nucleotides in the cell also allow *mec1Δ* mutants to survive, one idea is that the essential function of *MEC1* is to coordinate DNA synthesis with nucleotide availability (Desany *et al.*, 1998). It is possible that *rad3*$^+$ functions similarly, inhibiting origin firing when DNA replication is compromised. This model supposes that a normal cellular function of *rad3*$^+$ is to inhibit late origins in the presence of stalled DNA replication (or DNA damage), and that overexpression of *rad3*$^+$ increases this inhibition. It is difficult but not impossible to reconcile this model with the finding that Rad3p catalytic activity is not required for the phenotypes; perhaps *OPrad3*$^+$ inhibits origins in a way that does not involve phosphorylation of downstream substrates. For example, while

Rad3p may normally inhibit DNA replication by phosphorylating a downstream protein, high levels of Rad3p may inhibit the same protein by titrating it into nonfunctional complexes.

Another possibility is that *OPrad3*$^+$ prevents or interferes with the upregulation of nucleotide biosynthesis necessary for recovery from stalled DNA replication. Consistent with this idea, both conditions may require elevated levels of ribonucleotide reductase. It is believed that cells recover from HU treatment through the upregulation of RNR (Harris *et al.*, 1996). In addition, certain *S. cerevisiae* pol∂ alleles have been identified as "*crt*" mutants, because they constitutively activate RNR transcription (Zhou and Elledge, 1992). It is possible that some *pol∂* mutants have a greater requirement for upregulation of RNR than others. Thus, cells may respond to the defects caused by *cdc6* mutants and the HU arrest through a common mechanism, increasing nucleotide availability. *OPrad3*$^+$ may interfere with this cellular response.

If overexpression of *rad3*$^+$ does reduce the availability of nucleotides in the cell, then *OPrad3*$^+$ might be acting in opposition to the normal cellular function of *rad3*$^+$. While the normal role of *rad3*$^+$ may be to activate a factor required for upregulation of RNR activity, its overexpression may reduce the activity of this factor, by titrating it away from its normal protein complexes. Studies in *S. cerevisiae* support the idea that the *rad3*$^+$ homologue *MEC1* plays a role in regulating nucleotide levels. For example, *MEC1* is known to participate in a regulatory cascade that leads to the induction of *RNR* transcription during DNA damage or stalled DNA replication (Kiser and Weinert, 1996). *MEC1* may also increase the availability of nucleotides in additional ways that are not yet

understood. For example, Mec1p may act to relieve the posttranslational inhibition of RNR by Sml1p (Zhao *et al.*, 1998), although a direct regulatory link between Mec1p and Sml1p has yet to be demonstrated. Much less is known about the regulation of RNR in *S. pombe*, although it is known that RNR is upregulated in response to DNA damage or hydroxyurea arrest (Harris *et al.*, 1996). This model has a number of testable predictions. First, the two *cdc6* alleles which show the phenotype with *rad3*+ should be hypersensitive to inhibitors of nucleotides. In addition, increased expression of RNR may be able to suppress the synthetic dosage phenotype between *rad3*+ and *cdc6*. It is also possible that overexpression of *rad3*+ interferes with the induction of RNR transcription, which can be measured directly by Northern analysis.

Although the molecular mechanism behind the *rad3*+ overexpression phenotypes is uncertain at this time, we believe that future studies on these phenotypes will prove to be valuable for understanding how wild-type levels of *rad3*+ influence DNA replication.

Materials and Methods

Strains

Strains used in this study are listed in Table 6. These strains were transformed with various plasmids, which are listed in Table 7, depending on the experiment. Transformations were performed by electroporation (Prentice, 1992) or by a lithium acetate overnight procedure (Elble, 1992). In all cases, gene expression from the transformed plasmids was controlled by the thiamine-repressible promoter *nmt1*+(Basi *et al.*, 1993). Under the *nmt1*+ promoter, gene expression is induced approximately 80-fold when thiamine is removed from the

Table 6. *S. pombe* strains used in this study.

Strain Number	Genotype	Source/Reference
TE235	*leu1-32 h⁻*	
TE239	*leu1-32 h⁺*	
TE255	*rad27::ura4⁺ (chk1Δ) ade6-704 leu1-32 ura4-d18 h⁺*	T. Carr
TE459	*rad1::ura4⁺ leu1-32 his⁻*	(Sunnerhagen et al., 1990)
TE482	*hus1::LEU2 leu1-32 h⁻*	(Kostrub *et al.*, 1997)
TE606	*cdc18-K46 leu1-32 h⁻*	This study; (Kelly *et al.*, 1993)
TE694	*cdc19-P1 leu1-32 h⁻*	This study; (Forsburg and Nurse, 1994)
TE697	*cdc6-23 leu1-32 h⁻*	This study; (Iino and Yamamoto, 1997)
TE699	*cdc1-7 leu1-32 h⁺*	This study; (MacNeill *et al.*, 1996)
TE750	*cdc27-M57 leu1-32 h⁺*	This study
TE754	*cdc6-121 leu1-32 h⁻*	Stuart MacNeill
TE860	*cdc6-23 cdc2-3w leu1-32 h⁻*	This study
TE877	*cdc20-M10 leu1-32 h⁺*	L. Moynihan; (D'Urso and Nurse, 1997)
TE905	*polδts3 leu1-32 h⁻*	This study (Francesconi *et al.*, 1993)
TE907	*polδts2 leu1-32 h⁻*	This study (Francesconi *et al.*, 1993)
TE908	*polδts1 leu1-32 h⁺*	This study (Francesconi *et al.*, 1993)

TE926	*cdc6-23 cds1::ura4*$^+$ *leu1-32 ura4-D18 h*$^+$	This study
TE933	*cdc6-23 chk1::ura4*$^+$ *leu1-32 ura4-D18 h*$^+$	This study
TE981	*rad3::ura4*$^+$ *ura4-D18 h*$^+$	This study; (Bentley *et al.*, 1996)
TE982	*cdc6-121 ura4-D18 h*$^+$	This study
TE984	*cdc6-23 ura4-D18 h*$^-$	This study
TE985	*pol∂ts2 (cdc6) ura4-D18 h-*	This study
TE986	*pol∂ts3 (cdc6) ura4-D18 h*$^+$	This study
TE987	*pol∂ts1 (cdc6) ura4-D18 h*$^+$	This study
TE988	*rad3::ura4*$^+$ *ura4-D18 h*$^-$	This study
TE997	*rad3::ura4*$^+$ *pol∂ts2 ura4-D18 h*$^-$	This study
TE998	*rad3::ura4*$^+$ *cdc6-121 ura4-D18 h*$^+$	This study
TE999	*rad3::ura4*$^+$ *pol∂ts3 ura4-D18 h*$^+$	This study

Table 7. DNA plasmids used in this study.

Number	Plasmid	Source/Reference
pTE101	rep3x	
pTE157	rep1-*rad3*$^+$	(Bentley *et al.*, 1996)
pTE169	rep1-*rad26*$^+$	T. Carr
pTE446	rep1-*rad3-C725*	Chapter 2
pTE478	rep1-*rad17*$^+$	This study
pTE479	rep1-*rad9*$^+$	This study
pTE531	rep1-*cds1*$^+$	(Forbes *et al.*, 1998)
pTE567	rep1-*rad1*$^+$	C. Kostrub
pTE696	rep1-*rad3-N775*	Chapter 2
pTE716	rep1-*rad3-LZ*	Chapter 2
pTE743	rep1-*rad3-LZ-KD*	Chapter 2

media (Maundrell, 1990). Media and growth conditions were as previously described (Moreno *et al.*, 1991). Transformed strains were routinely maintained in minimal EMM media containing 2μM thiamine. To induce expression of checkpoint proteins, cultures were resuspended in media lacking thiamine. The *nmt* promoter is fully derepressed approximately 16 hours after the removal of thiamine; it takes approximately 20 hours to repress an active promoter with the addition of thiamine (Maundrell, 1990). Fluorescence microscopy was performed using a Zeiss Axiophot microscope and a Photonic Microscope Image Processor C1966 (Hamamatsu Photonic Sys. Corp., Bridgewater, NJ).

Description of plasmids

The rep1-*rad17*$^+$ plasmid (pTE478) was generated by isolating the *Nde*I-*Sal*I insert from the rep41-*rad17*$^+$ plasmid (a generous gift of A. Carr), and ligating it into the similarly cut rep1 backbone (pTE102). To create the rep1-*rad9*$^+$ plasmid, the *Pst*I-*Sac*I fragment from rep2-*rad9*$^+$ plasmid (pTE575), which contains the *nmt1*$^+$ promoter, the *rad9*$^+$ gene, and the *nmt* polyA sequences, was subcloned into the similarly cut rep1 (pTE102) backbone.

Synthetic Dosage Lethality Assays

Single colony transformants were streaked onto minimal (EMM) plates containing thiamine, which represses gene expression from the *nmt1*$^+$ promoter, and grown at 25°C for 2-3 days. These plates were then replica plated onto minimal (EMM) plates lacking thiamine. The synthetic dosage lethality was scored by visually assessing the length of the cells microscopically after 3 days of

growth on plates lacking thiamine. Alternatively, cells were inoculated into a thiamine-containing liquid EMM media starter culture. Cells were washed with water and reinoculated into EMM media lacking thiamine. After approximately 20-24 hours, the cultures were then rediluted in EMM to maintain the cultures in early-mid log phase. The synthetic dosage phenotype was clearly apparent after approximately 45-50 hours after induction of the *nmt*$^+$ promoter. Cells were fixed in cold 70% ethanol, and stored at -20°C. Later, cells were stained with the DNA dye DAPI and examined by fluorescence microscopy.

Viability Assessments

To assess the viability of various cultures, cell number was counted using a haeomocytometer. Cells were diluted in water, sonicated briefly, and approximately 500 cells were plated onto duplicate plates containing 2 μM thiamine. Pictures of colonies were taken after 5 days of growth.

Pulsed Field Gel Electrophoresis

Plug preparation was as described (Tanaka *et al.*, 1999). Cell number was measured using a haemocytometer. Cells were washed twice in CSE buffer (20 mM citrate-phosphate, pH 5.6, 1.2 M sorbitol, 40 mM EDTA) containing 150 mM B-mercaptoethanol, and then resuspended in 10 ml CSE buffer containing 30 mM B-mercaptoethanol and 15 mg Zymolyase-20T (Seikagaku Corporation). After digestion for 1 hour at 37°C, cells were pelleted and resuspended at 8 x 10^8 cells/ml in TSE (10 mM Tris-HCl pH 7.5, 0.9 M sorbitol, 45 mM EDTA). An equal volume of 1% low-melt agarose in TSE was added, and this suspension was dispersed into plug molds (Bio-Rad Disposable Plug Molds #170-3713). Plugs

were then incubated in lysing buffer (0.25M EDTA, 50 mM Tris-HCl pH 7.5, 1% SDS) for 90 minutes at 55°C, and transferred to NDS buffer (0.5 M EDTA pH 9.5, 1% lauryl sarcosine) containing 0.5 mg/ml proteinase K, and incubated for 48 h at 55°C. Plugs were stored in 20% NDS buffer at 4°C, and equilibrated with 1X TE before loading. Two plugs were loaded in each well. A 0.6% chromosomal grade agarose (SeaKem Gold, FMC Bioproducts) gel was run for 72 hours in 0.5X TAE buffer in a BioRad CHEF Mapper apparatus at 14°C, with an angle of 120°, a pulse time of 30 minutes, and a voltage gradient of 1.5 V/cm. The gel was stained with 0.5 μg/ml ethidium bromide.

Hydroxyurea Time Course/FACS analysis

Overnight cultures of transformed strains were grown up in minimal media containing 2 μM thiamine to maintain repression of the *nmt1*+ promoter. To induce expression from the *nmt1*+ promoter, these cultures were diluted into media lacking thiamine and grown for 20 hours to an O.D.595 between 0.1 and 0.2. 10 mM HU was then added, and the cultures were incubated at 29°C with shaking. Samples were taken every two hours and fixed with cold 70% ethanol. These samples were prepared for FACS (fluorescence activated cell sorting) analysis as described (Sazer and Sherwood, 1990), except that the ethanol-fixed cells were stored at -20°C prior to processing for propidium iodide staining. FACS analysis was performed with a FACS Calibur cytometer and CellQuest version 3.1f software (Becton Dickinson, San Jose, CA). 10,000 events were counted for each sample. Ethanol-fixed cells were also stained with DAPI to examine cells by fluorescence microscopy and count aberrant mitoses. For each experiment, at least 100 cells were counted for the 0 hour time point, and at least

200 cells were counted for the 10 hour time point. The results from two independent experiments were averaged.

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