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Characterization of *Saccharomyces cerevisiae dna2* Mutants Suggests a Role for the Helicase Late in S Phase

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The TOR proteins, originally identified as targets of the immunosuppressant rapamycin, contain an ATM-like "lipid kinase" domain and are required for early G1 progression in eukaryotes. Using a screen to identify *Saccharomyces cerevisiae* mutants requiring overexpression of Tor1p for viability, we have isolated mutations in a gene we call *ROT1* (requires overexpression of Tor1p). This gene is identical to *DNA2*, encoding a helicase required for DNA replication. As with its role in cell cycle progression, both the N-terminal and C-terminal regions, as well as the kinase domain of Tor1p, are required for rescue of *dna2* mutants. *Dna2* mutants are also rescued by Tor2p and show synthetic lethality with *tor1* deletion mutants under specific conditions. Temperature-sensitive (Ts) *dna2* mutants arrest irreversibly at G2/M in a *RAD9*- and *MEC1*-dependent manner, suggesting that Dna2p has a role in S phase. Frequencies of mitotic recombination and chromosome loss are elevated in *dna2* mutants, also supporting a role for the protein in DNA synthesis. Temperature-shift experiments indicate that Dna2p functions during late S phase, although *dna2* mutants are not deficient in bulk DNA synthesis. These data suggest that Dna2p is not required for replication fork progression but may be needed for a later event such as Okazaki fragment maturation.

INTRODUCTION

Helicases are a class of nucleoside triphosphate (NTP)-dependent enzymes that catalyze the unwinding of double-stranded nucleic acids by disrupting the hydrogen bonds between complementary base pairs (bp). DNA helicases that unwind duplex DNA are essential for DNA replication, recombination, and repair, while RNA helicases are required for transcription, pre-mRNA processing, regulation of RNA stability, ribosome assembly, and protein translation. All helicases contain seven characteristic regions of similarity, termed I, Ia, and II–VI (Gorbalenya *et al.*, 1989). Motifs I and II comprise a nucleotide binding/hydrolysis domain, and motif II contains the so-called DEAD, DEAH, or DEXH boxes that are found in a variety of viral and eukaryotic RNA helicases (Schmid and Linder, 1992). The exact function of the other motifs is not clear, although each region is presumed to represent a functional domain having a role in either NTP binding and/or hydrolysis as well as in catalyzing the separation of duplex nucleic acid.

Although originally envisioned as being required for separation of double-stranded template DNA to allow advancement of the replication fork, it is now appreciated that DNA helicases are required for a variety of processes, including that of Okazaki fragment maturation and repair of replicative errors (Matson *et al.*, 1994). In budding yeast, many helicases have been identified genetically or biochemically that are thought to have a role in DNA replication (Li *et al.*, 1992a, 1992b; Shimizu and Sugino, 1993; Schulz and Zakian, 1994; Biswas *et al.*, 1995; Budd and Campbell, 1995). Despite this, definitive roles for these enzymes in either leading or lagging strand synthesis have not yet emerged.

Recently, Campbell and co-workers identified the Dna2p protein as an essential helicase required for

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DNA synthesis in *S. cerevisiae* (Budd *et al.*, 1995; Budd and Campbell, 1995). They demonstrate that the enzyme catalyzes separation of DNA:DNA hybrids in vitro and speculate that the 3′→5′ polarity of the enzyme is consistent with a role in leading strand synthesis. Recent evidence suggests that Dna2p interacts functionally and physically with the product of the *RAD27/RTH1* gene (Budd and Campbell, 1997). Because the mammalian homolog of this gene product, FEN-1, is required for Okazaki fragment processing in vitro, the authors have suggested that Dna2p (via interaction with the yeast FEN1 gene product) might, in fact, have a role in lagging strand DNA synthesis (Goulian *et al.*, 1990; Turchi *et al.*, 1994). However, there are no clear data to support a role for Dna2p in either leading or lagging strand synthesis.

Rapamycin is a potent macrolide immunosuppressant that inhibits G1 progression in both mammalian and yeast cells (Schreiber, 1991; Terada *et al.*, 1993; Barbet *et al.*, 1996). The TOR proteins, which include yeast Tor1p and Tor2p and the mammalian homologues FRAP/mTOR/RAFT/RAPT1, were originally identified by both biochemical and genetic methods as the functionally relevant targets of rapamycin (Heitman *et al.*, 1991; Brown *et al.*, 1994; Chiu *et al.*, 1994; Sabatini *et al.*, 1994; Sabers *et al.*, 1995). These proteins contain a lipid kinase domain found in phosphatidyl inositol (PI) kinases, enzymes that are required for the production of PI-derived second messengers (Carpenter and Cantley, 1990). However, the TORs belong to the so-called phosphatidyl inositol kinase (PIK)-related family of kinases, whose members have roles in a variety of processes such as cell cycle progression, meiotic and V(D)J recombination, chromosome maintenance and repair, and DNA checkpoint signal transduction (Keith and Schreiber, 1995). It is postulated that the TORs, like other PIK-related family members, such as DNA-PK, function as protein kinases rather than (or in addition to) lipid kinases (Hunter, 1995; Keith and Schreiber, 1995). While some groups have reported associated PI 4-kinase activity with immunoprecipitated Tor2p and RAFT1 (Cardenas and Heitman, 1995; Sabatini *et al.*, 1995), others have failed to do so, and it is unclear whether this activity is intrinsic to or associated with the TOR proteins.

Multiple studies implicate the TOR/FRAP proteins in the control of protein translation. Rapamycin blocks protein synthesis in yeast by almost 90% (Drebot *et al.*, 1987), while the effect in mammalian cells is cell type-specific, ranging from 10 to 75% (Terada *et al.*, 1994; Giasson and Meloche, 1995). In addition, TOR/FRAP activity is required for activation of p70 S6 kinase, the physiologic regulator of phosphorylation of the S6 ribosomal subunit that is believed to have a role in the control of protein translation in higher eukaryotes (Hershey, 1989; Chung *et al.*, 1992). In addition, rapamycin blocks initiation of translation of transcripts containing 5′ polypyrimidine tracts (Jefferies *et al.*, 1994). More recently, it has been shown that rapamycin inhibits the growth factor-induced up-regulation of eIF-4E activity, a factor required for the initiation of translation of all 5′ capped transcripts (Graves *et al.*, 1995; Lin *et al.*, 1995).

However, it is not clear that the only role for the TOR proteins is to regulate protein translation. That is, the TOR proteins may be involved in the control of a wide variety of events that are required to execute a cellular response to an environmental stimulus, one of these being modulation of the translational apparatus. For example, yeast treated with rapamycin display all of the characteristics of starved cells (Barbet *et al.*, 1996). In addition, the yeast TOR proteins have been implicated in the control of the activity of Sit4p, a type 2A phosphatase required for G1 progression (Di Como and Arndt, 1996). Control of phosphatase function provides a potential mechanism for how the TORs might control a pleiotropic array of responses, since phosphatases are involved in a variety of distinct cellular processes. Recent evidence suggests that Tor2p is localized to the vacuole and plays a role in the fidelity of vacuolar segregation to mother and daughter cells (Cardenas and Heitman, 1995). More recently, Hall and colleagues have shown a role for Tor2p in the regulation of Rho-like guanosine triphosphatases and the actin cytoskeleton (Schmidt *et al.*, 1996, 1997).

Regardless of their cellular function, it is still unknown what mechanisms (if any) regulate the TORs or upon what substrates these putative kinases act. In a screen designed to identify genes that operate in the TOR-signaling pathway, we have isolated mutants that are dependent upon overexpression of Tor1p for viability. One such class of yeast were novel *dna2* mutants. In this paper we describe the isolation and characterization of these mutants and present the results of genetic experiments designed to illuminate the nature of the interaction between Tor1p and Dna2p. In addition, we describe the construction of temperature-sensitive (Ts) mutants that demonstrate that Dna2p acts late in S phase to perform an essential function that is consistent with a role in lagging strand DNA synthesis.

MATERIALS AND METHODS

Strains, Plasmids, and Media

The composition of rich glucose medium (YPD), rich galactose medium (YPGal), synthetic minimal medium (SD), synthetic complete medium (SC), and liquid sporulation medium (Spo) was as described (Sherman, 1991). YPD and YPGal media were adjusted to pH 6.0 with 1 N HCl. Synthetic plates containing 5-fluoroorotic acid (5-FOA) (PCR, Gainesville, FL) were made as described (Sikorski and Boeke, 1991). SD+canavanine plates were prepared by supplementing SD with amino acids and nucleotides dictated by strain auxotrophies as well as 60 g/l canavanine (Sigma Chemical, St. Louis, MO). All solid medium contained 2% agar except the YPGal

plates used in the original sectoring screen, which contained 1.8% agar and 100 mg/ml ampicillin (Sigma) to facilitate analysis by spreading colonies and to prevent bacterial contamination. α Factor (Sigma) was added to media from a 1 mg/ml stock in 10 mM HCl (stored at −20°C) to a final concentration of 5 μg/ml. Hydroxyurea (HU) (Sigma) was added to YPD to a final concentration of 0.2 M and sterile filtered before use. Nocodazole (Sigma) was added from a 3 mg/ml stock in dimethylsulfoxide (stored at −20°C) to a final concentration of 15 μg/ml.

Yeast mating, sporulation, and tetrad dissection were performed according to standard methods (Sherman and Hicks, 1991). Yeast were transformed by the lithium acetate method (Ito *et al.*, 1983).

pYDF18 is an N-terminal hemagglutinin (HA)-tag expression vector derived from PG-1 (a 2μ, *TRP1* -based plasmid utilizing the G6PD promoter) (Schena *et al.*, 1991). pYDF66, an HA-Tor1p expression vector, was made by cloning the full-length *TOR1* gene into pYDF18. The following genomic restriction sites within *TOR1* were used to make amino- and carboxyl-terminal deletions of Tor1p using pYDF66 as a template: *Spe*I (Δ1–99); *Nde*I (Δ1–416); *Xho*I (Δ1–724); *Sac*II (Δ1–1198); *Nco*I (Δ1–1764); *Acc*I (Δ1–1882); *Pfl*M1 (Δ1–2067); *Bsi*W1 (Δ1–2225); *Aat*II (Δ2441–2470); *Bgl*II (Δ2260–2470). Kinase-defective mutants of Tor1p (Zheng *et al.*, 1995) were cloned into pYDF66 as C-terminal *Bsi*W1/*Sal* fragments. pYDF77 is *GAL1*p→*TOR1* on red/white indicator plasmid, made by cloning a *Nhe*I/*Sal*I fragment containing the *TOR1* gene into pMW29, a *URA3*-based centromeric plasmid containing the *ADE3* gene and the *GAL1* promoter with a MCS downstream (Zieler *et al.*, 1995). pYDF88 is a *LEU2*-based form of pYDF77 without the *ADE3* gene and was used for plasmid shuffling as described (Zieler *et al.*, 1995). PYDF125 (a generous gift of S. Schreiber, Harvard University, Boston, MA) is an HA-tagged form of *GAL1*p→*TOR1* in the *LEU2*-based vector, PRS315 (Sikorski and Hieter, 1989). pYDF117 was created by cloning a *Cla*I/*Pst*I genomic fragment containing the *DNA2* gene into *Cla*I/*Pst*I-digested PRS304, a *TRP1*-based integration vector (Sikorski and Hieter, 1989). The *TOR1* disruption plasmid (pYDF79) was made by ligating a *Bam*HI/*Xho*I fragment (nucleotides 988–2172) and a *Sac*II/*Bam*HI fragment (nucleotides 3600–5047) simultaneously into *Xho*I/*Sac*II-digested PRS304 (Sikorski and Hieter, 1989) to create a disruption fragment that removes amino acids 330–724 of Tor1p and replaces them with the *TRP1* gene and pBluescript (Stratagene, La Jolla, CA).

CB018 is a protease-deficient strain constructed by C. Brenner (Brandeis University, Waltham, MA). YDF92-YDF102 are derived from either TW397 (wild-type), TW398 (*rad9*), or TW308 (*mec1–1*) (Weinert *et al.*, 1994). Strain YDF20 (*tor1*-Δ10) was created by transforming YMW1 with *Bam*HI-digested pYDF79 and selecting for *TRP*+ cells (deletion confirmed by Southern blotting). Strain YDF104 was constructed by mating DBY1034 with DBY1511 (D. Botstein, Stanford University, Stanford, CA). YDF105 was constructed by genomic replacement of the *DNA2* gene with the *dna2–22* temperature-sensitive (Ts) allele (see below) in strain DBY1034 and mating this strain with DBY1511. YDF106 was constructed by genomic replacement of the *DNA2* gene with the *dna2–212* Ts allele in both strains DBY1034 and DBY1511 and then mating these two strains. Unless otherwise stated, the *dna2–22* Ts mutation was introduced into all strain backgrounds by integrative transformation and never by crossing strains (see below). Thus, all comparisons between *dna2* mutant cells and wild-type cells are between strictly isogenic strains.

Sectoring Screen for Mutants Requiring Tor1p Overexpression

To identify mutants requiring a Tor1p overexpression plasmid for viability, we performed a red/white sectoring assay utilizing yeast expression plasmids and strains exactly as described (Zieler *et al.*, 1995). Briefly, the strain background (YMW1) is *ade2 ade3*, which produces white colonies on rich media, but cells carrying a plasmid with the *ADE3* gene ("indicator plasmid") will form red colonies. During growth, the small percentage of cells that randomly lose the plasmid give rise to white "sectors" within an otherwise red colony. Any colony that is all red represents either yeast that have permanently converted to *ADE3*+ phenotype (via a plasmid recombination or gene conversion event) or yeast that require sequences on the plasmid for viability. By including an expression cassette for Tor1p on this plasmid, it is thus possible to screen for mutants that require this expression cassette (i.e., overexpression of Tor1p) for viability. The YMW1/2 strains were transformed with the a *GAL1*p→Tor1p overexpression indicator plasmid (pYDF77) and mutagenized with ethylmethane sulfonate (Sigma) to 50% viability as described (Lawrence, 1991). This plasmid gives functional overexpression of Tor1p as assessed by a 5-fold increase in rapamycin resistance when cells are grown in YPGal (our unpublished results). Mutagenized cells (stored in water at 4°C) were plated on 150-mm YPGal plates (to allow Tor1p overexpression) at a density of 500/plate and incubated at 30°C for 5–7 d. A total of 80,000 colonies were screened. Potential positive nonsectoring colonies were then screened for true Tor1p overexpression dependence as described (Zieler *et al.*, 1995). Briefly, this included requiring that mutants: 1) die or grow very slowly on YPD; 2) die or grow very slowly on FOA-containing plates that select against the indicator plasmid; 3) are able to lose the indicator plasmid on YPGal if provided with an alternate *GAL1*p→*TOR1* overexpression plasmid (pYDF88) not containing the *ADE3* gene. All mutants were recessive and found to represent single gene defects as assessed by mating and tetrad analysis (our unpublished results).

Cloning of the ROT1/DNA2 *Gene*

Mutant 2–71 (*rot1–1*) containing pYDF88 (*GAL1*p→*TOR1*) was grown overnight in YPGal and transformed with 4 μg of a genomic DNA library in YCP50 (Rose *et al.*, 1987), and transformants were selected on SCGal-ura plates (~250,000 colonies total). After 2 d at 30°C, transformants were replica plated to YPD plates for 1 d at 30°C. Colonies demonstrating growth on YPD (120 total) were picked and restreaked on glucose-containing FOA plates for 2 d. Of these, two colonies that showed virtually no growth were then picked from the original SCGal-ura plates and plasmid DNA was extracted (Strathern and Higgins, 1991). Upon restriction mapping, it was apparent that both of the yeast colonies contained the identical library plasmid, and dideoxy sequencing analysis was carried out as described (Sambrook *et al.*, 1989).

To confirm that a mutation in *DNA2* is the relevant alteration in the *rot1–1* mutant, a strain was constructed that contained the *TRP1* gene integrated at the *DNA2* locus by transforming yeast with a plasmid containing the *TRP1* and *DNA2* genes (pYDF117) that had been linearized by cutting with a single *Nde*I site within the *DNA2* gene; this generated a genomic duplication of the *DNA2* genes separated by the *TRP1* gene (confirmed by Southern blotting). This strain (YDF26) was mated to the *rot1–1* mutant, and, upon dissection of 14 tetrads onto YPD plates, all viable spores were found to be *TRP1*$^+$, indicating that the mutation is tightly linked to *DNA2* (our unpublished results).

Locating dna2 *Mutations by Gap Repair*

The location of the *dna2–20* and *dna2–21* mutations was determined by a gap repair method (Rothstein, 1991). This analysis was performed using gapped variants of a *DNA2*-containing *Cla*I/*Pst*I genomic fragment cloned into PRS314 (Sikorski and Hieter, 1989) as templates for repair. The *dna2* mutants carrying pYDF88 were transformed with these gapped variants and selected on SCGal-leu-trp (selecting for Tor1p overexpression as well as the repaired plasmid). Individual colonies (20) were picked and restreaked on YPD at 30°C and assayed for growth. The presence of only mutant colonies (i.e., inability to grow on YPD) was inferred to indicate that a particular template was missing the region mutated. This analysis located the presence of both the *dna2–20* and *dna2–21* mutations between a

Bsm1 and PpuM1 site spanning amino acids 1076–1197. This region was amplified from mutant genomic DNA by the polymerase chain reaction, and sequence analysis demonstrated that each mutant contained only one mutation that would result in an amino acid alteration (our unpublished results).

Construction of dna2 *Mutants*

The *dna2-Δ20* disruption strain was constructed by replacing nucleotides 366-4215 of the genomic *DNA2* sequence with a *Bam*HI fragment containing the *TRP1* gene by using the one-step gene replacement technique (Rothstein, 1991) with a 2.7-kb *Cla*I/*Pst*I disruption fragment. Diploid transformants were selected on SC-trp and disruptants screened by polymerase chain reaction with the following primers: ROT-1A (5′-GATCGTCGACGGGATCCCGAATTCCATGCCCGGAA-CGCCACAGAAG-3′); TRP-1B (CACGTGCTCAATAGTCACCAA-TGC). Of 13 screened, six were positive and one of these was chosen and called YDF27.

dna2 conditional alleles were isolated by a plasmid shuffle technique as described (Sikorski and Boeke, 1991). The recipient host strain was constructed by transforming YDF27 with pYDF113, dissecting tetrads, and selecting for a *dna2* disruptant (i.e., *TRP1*$^+$). Plasmid pYDF132 (containing a *DNA2* genomic fragment in a *LEU2*-based centromeric vector) was the template for mutagenesis with hydroxylamine (Sigma). The efficiency of mutagenesis was assessed by the frequency of loss-of-function mutations in the *LEU2* gene on the plasmid. Mutagenesis to 4–5% loss of leucine prototrophy (assessed in a *leuB6* HB101 bacterial strain) was chosen, and this DNA was transformed directly into the recipient strain. Approximately 12,500 transformants were screened, resulting in 10 strains showing wild-type growth at 30°C and no growth at 37°C as assessed by colony-forming ability (our unpublished results). One of these, *dna2–22*, was selected and integrated into the yeast genome using the pop-in/pop-out gene replacement technique (Scherer and Davis, 1979).

Preparation of Anti-Dna2p Antibodies

Bacteria were transformed with a GST-ROT1p fusion constructed by cloning a *Nco*I/Bsm1 fragment of the *DNA2* gene encoding aa 121–251 into the GEX2T expression vector (Smith and Johnson, 1988). GST fusion proteins were prepared and purified as described (Smith and Johnson, 1988) and injected into rabbits. Rabbits were boosted biweekly, and antisera were affinity purified using GST-Dna2p CNBr-activated columns (Pharmacia, Washington, DC) as described (Harlow and Lane, 1988). The reactivity of the antisera was assessed by comparing immunoblots of extracts from wild-type cells with those from cells overexpressing the Dna2p protein from the *GAL1* promoter. A doublet representing polypeptides of molecular masses of approximately 170 and 190 kDa, which was detected only with immune serum and was expressed at approximately 20-fold excess over that of control cells, was assumed to represent the Dna2p polypeptide(s) (our unpublished results).

Preparation of Yeast Whole Cell Extracts

Log-phase yeast cells were spun down and washed in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.2% TritonX-100), and 100 μl of cells were spun into each Eppendorf tube and resuspended in 150 μl lysis buffer with protease inhibitors (Boehringer Mannheim, Indianapolis, IN) (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 μg/ml antipain, 10 μg/ml pepstatinA, 2 mM benzamidine, 10 μg/ml aprotinin, 65 μg/ml L-1-tosylamide-2-phenylethylchloromethyl, 65 μg/ml TLCK). Two hundred microliters of glass beads (425- to 600-μm, Sigma) were added, and the tubes were vortexed twice for 5 min. Cell lysis was defined by >80% of the cells being trypan blue permeable. The tubes were punctured in the bottom with a 26.5-gauge needle, and supernatants were spun into clean tubes at 2000 × *g* for 30 sec. The supernatants were then spun at 15,000 × *g* for 5 min, transferred to TLA.100.2 tubes, and spun at 100,000 × *g* for 20 min at 4°C in a Beckman TL-100 ultracentrifuge (Beckman, Fullerton, CA). Supernatants were assayed for protein concentration by the Bradford assay and either used immediately or made 30% (vol/vol) in glycerol and frozen at −80°C.

Immunoprecipitation and Immunoblotting

Protein A-Sepharose (Pharmacia) beads were washed several times in lysis buffer (see above) and, for each sample, 15 μl packed beads were incubated with 1 mg extract protein plus proper amount of antibody (1/30 μl of anti-Dna2p affinity purified antibodies or 1 μl 12CA5 anti-HA ascites), and the total volume was brought up to 500 μl in lysis buffer. Tubes were rotated at 4°C for 2 h and then spun for 1 min at 10,000 × *g* and washed three to four times in lysis buffer with protease inhibitors. After the final wash, pellets were resuspended in 1× SDS sample buffer and boiled 5 min. Immunoprecipitates or whole extracts were separated using SDS-PAGE and transferred to nitrocellulose as described (Harlow and Lane, 1988; Sambrook *et al.*, 1989). Immunoblotting was carried out essentially as described using affinity-purified anti-Dna2p antibodies (1:10,000), anti-HA ascites (1:1000), anti-actin antisera (1:10,000) (a kind gift of D. Botstein, Stanford University). Horseradish peroxidase (HRP)-conjugated reagents include affinity-purified goat anti-rabbit-HRP antibodies (1:3000) (Jackson Immunoresearch, West Grove, PA), rabbit anti-mouse-HRP antibodies (1:1500) (Jackson) (Harlow and Lane, 1988), and Protein A-HRP (Jackson). Protein levels were quantified using a model 300A densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Flow Cytometry

Haploid yeast were grown under the appropriate experimental conditions to approximately 1×10^7 cells/ml. Cells (5–10 ml) were sonicated for 20 sec on ice with a microtip sonicator on setting 2 to disperse clumps. Cells were washed once with Tris buffer (50 mM Tris-HCl, pH 7.5) and resuspended in 1.5 ml distilled water to which 3.5 ml 100% ethanol were added (70% final) and incubated for 1 h at room temperature. Cells were washed with 5 ml Tris buffer and resuspended in 1 ml of RNase (Sigma) buffer (10 mg/ml ribonuclease A in sodium acetate, pH 5.0, diluted 1:10 into Tris buffer). Cells were spun down and resuspended in 1 ml of freshly made pepsin (Sigma) solution (5 mg/ml in 0.05 N HCl) for 5 min at room temperature. Cells were spun down and washed in 2 ml propidium iodide (PI, Sigma) staining solution (0.05 mg/ml PI diluted into 200 mM Tris, pH 7.5, 200 mM NaCl, 70 mM $MgCl_2$) and incubated for 1 h at room temperature and then overnight at 4°C in the dark. Cells were washed into 3 ml Tris buffer and analyzed on a FACScan machine (Becton-Dickinson, Lincoln Park, NJ). Twenty thousand events were analyzed for each sample.

α Factor Synchronization/Release Protocol

Log-phase cells were resuspended in 1 ml YPD at OD 0.05, and α factor was added to 5 μg/ml and cells were cultured for 2.5 h at 30°C. Cell cycle arrest was monitored by accumulation of >95% unbudded cells as visualized by phase contrast microscopy. In some experiments cells were then placed in a 37°C shaking water bath, and 5 μg of α factor were added every hour for 1–3 h. Next, cells were spun down in an Eppendorf tube, washed twice in YPD, and diluted 1:20 into prewarmed YPD with 0.05 mg/ml pronase (Sigma) and 0.05 mg/ml proteinase K (Sigma) and cultured at the appropriate temperature. If indicated, HU and nocodazole were added at this time. At different times after α factor release, 75 μl were removed from the cultures and diluted into 425 μl cold YPD on ice to immediately halt cell cycle progression. Cells were sonicated with a microtip sonicator, diluted, and plated on duplicate YPD plates for 2 d at 30°C. A minimum of 150 colonies was counted for each time point.

Mitotic Recombination and Chromosome Loss Assay

Recombination and chromosome loss frequencies were quantified using the selection scheme of Hartwell and Smith (1985), essentially as described (Holm *et al.*, 1989).

4,6-Diamidino-2-Phenylindole (DAPI) Staining

Cells were grown to an OD between 0.1 and 0.2 at the appropriate temperature before addition of 10% ultrapure formaldehyde (Polysciences, Inc., Warrington, PA) to a final concentration of 4%. After 10 min, cells were resuspended in 5 ml phosphate buffer (40 mM KPO_4, pH 6.5, 0.5 mM $MgCl_2$) with 4% formaldehyde and incubated for 1 h at the appropriate temperature. Next, cells were washed twice in phosphate buffer and resuspended in mounting medium, consisting of 10 mg/ml *p*-phenylenediamine (Sigma) in PBS, pH 9.0, in 90% glycerol with 20 ng/ml DAPI (4′, 6′-diamidino-2-phenylindole) (Accurate Chemical and Scientific Corp., Westbury, NY). Cells were visualized with Nomarski optics using a Zeiss Axioskop (Carl Zeiss, Thornwood, NY) with a 100×, 1.4 plan-neofluor lens.

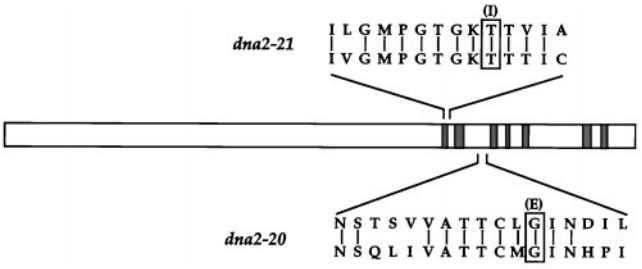

Figure 1. Identification of the mutations in *dna2–20* and *dna2–21* alleles. Schematic drawing of the *DNA2* sequence with the location of the seven helicase domains shown hatched and drawn to scale. Regions that contain the mutations are enlarged. The yeast and human *DNA2* sequences are shown on the top and bottom, respectively, with conserved residues connected by bars. The mutated residue is boxed and the resulting amino acid shown in parentheses.

RESULTS

A Screen for Mutants Requiring Elevated Levels of Tor1p

We have utilized a sectoring assay (Hartwell and Smith, 1985; Sanchez *et al.*, 1996) that employs a *TOR1*$^+$ strain and a *GAL1*p→*TOR1* indicator plasmid to identify mutants that require the indicator plasmid (i.e., Tor1p overexpression) for optimal growth. On media containing galactose (YPGal) this plasmid gives functional overexpression of Tor1p when compared with cells grown with the plasmid in glucose (YPD), which suppresses Tor1p expression, and thus mutants were screened that require the plasmid on YPGal but cannot survive (or grow poorly) on YPD. This screen not only identifies mutants absolutely requiring Tor1p overexpression for viability but also those for which increased levels of Tor1p provide a relative growth advantage. Thus, a *tor1* deletion mutant fails to display sectoring under these conditions, presumably due to the slight growth advantage of *TOR1*$^+$ over *tor1*$^-$ cells in our strain background (Figure 4A; our unpublished results). A screen of ~80,000 mutagenized yeast yielded 10 recessive mutants. Seven of these were *tor1* mutants, as assessed by linkage to a *tor1* deletion mutant (our unpublished results). Of the remaining three mutants, one was found to be in the *TOR2* gene (our unpublished results). Since the purpose of this study was to identify novel genes in a putative TOR-signaling pathway, these mutants will not be described further.

On the basis of complementation and linkage analysis, the final two mutants were found to be in the same gene which we named *ROT1* (requires overexpression of Tor1p). This gene was cloned by complementation of the recessive *rot1–1* mutant, and DNA sequencing revealed that the insert was a fragment from the right arm of chromosome VIII (Johnston *et al.*, 1994). This insert complemented the growth defect of both *rot1* mutants, and a deletion analysis of this fragment demonstrated that a 4.5-kb open reading frame encoded the complementing activity (our unpublished results). BLAST analysis (Altschul *et al.*, 1990) revealed that this open reading frame corresponded to *DNA2*, a gene recently identified as being required for DNA replication in yeast and found to encode a protein with 3′→5′ DNA helicase activity (Budd *et al.*, 1995; Budd and Campbell, 1995). As expected, the carboxyl-terminal one-third of the protein (amino acids 1072–1434) contains a region that closely matches a heptapartite consensus sequence that defines a superfamily of proteins with helicase activity (Schmid and Linder, 1992). That this gene may perform an essential function in all eukaryotic cells is underscored by the fact that a human cDNA clone has been identified that displays 34% overall identity (45% within the carboxyl-terminal 400 amino acids) to the yeast gene and is likely to encode the human homologue of *DNA2* (Budd and Campbell, 1995). To avoid confusion, we have renamed the *rot1–1* and *rot1–2* mutants *dna2–20* and *dna2–21*, respectively.

Since the sequence of *DNA2* revealed at least one functional domain, we wished to determine whether the mutations in the *dna2–20* and *dna2–21* alleles are within this putative helicase region. The approximate location of the mutations was determined by a gap repair method and found to be similar in both mutants (see MATERIALS AND METHODS). Sequence analysis of this region revealed that the *dna2–21* mutation is a C→T transition at nucleotide 3233, causing a thr_{1078}→ile_{1078} change within region I, a domain known to comprise a critical part of the NTP-binding fold (Figure 1) (Walker *et al.*, 1982). The *dna2–20* mutation is a G→A transition at nucleotide 3500, causing a gly_{1167}→glu_{1167} change in an area between regions II and III of the consensus helicase domain. These mutations are consistent with the fact that ethylmethane sulfonate mutagenesis exclusively causes transitions

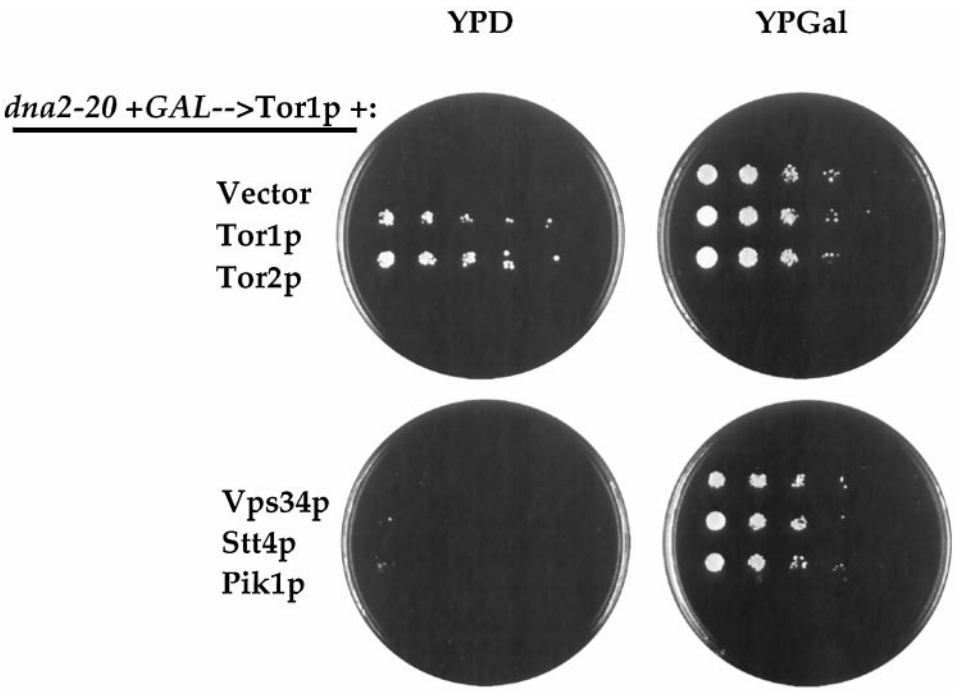

Figure 2. Rescue of *dna2–20* is TOR-specific. The *dna2–20* mutant carrying pYDF88 (*GAL1*p→*TOR1*) was transformed with the following plasmids: pYDF18 (vector), pYDF66 (Tor1p), pJK3–3 (Tor2p) (Kunz *et al.*, 1993), 324.34 (Vps34p) (Herman and Emr, 1990), YEp352-STT4 (Stt4p) (Yoshida *et al.*, 1994), and YEp352-PIK1 (Pik1p) (Flanagan *et al.*, 1993). All of these plasmids are 2μ-based and contain the genes under control of their endogenous promoters, except for the Tor1p expression construct that utilizes the GPD promoter (see MATERIALS AND METHODS). Transformants were selected on SCGal-leu minus the appropriate amino acid to also select for the various plasmids. Cells were scraped off plates (representing ~100 colonies), resuspended in water, sonicated, and spotted onto YPD and YPGal plates. Ten thousand cells were spotted on the leftmost region with successive fivefold dilutions moving to the right. The plates were incubated for 3 d at 30°C and photographed.

at G·C sites (Kohalmi and Kunz, 1988). Additionally, both of these mutations are in residues conserved between the yeast and putative human *DNA2* genes (Budd and Campbell, 1995), and they occur within a region predicted to be essential for the helicase function of Dna2p. Indeed, mutations in the invariant lysine$_{1080}$ in the NTP-binding fold of region I ablate the helicase and biological activity of *DNA2* (Budd *et al.*, 1995).

The dna2–20 *Mutant is Specifically Rescued by Tor1p and Tor2p*

Because the purpose of this study was to learn more about the function that is shared by Tor1p and Tor2p, we tested the ability of Tor2p to rescue the *dna2–20* mutant. In these experiments, the *dna2–20* mutant carrying *GAL1*p→*TOR1* is transformed with the overexpression plasmid to be tested and plated on either YPD (experimental) or YPGal (as a control for plating efficiency). As shown in Figure 2, *TOR1* provided on a high-copy plasmid under control of the G6PD promoter effectively rescues the mutant on glucose-containing media, ruling out carbon source artifacts contributing to the original rescue with the *GAL1*p→*TOR1* plasmid. In addition, a genomic fragment containing *TOR2* (and its endogenous promoter) provided on a multicopy plasmid efficiently rescues the *dna2–20* mutant (Figure 2). However, a similar multicopy vector containing a *TOR1* genomic fragment is unable to suppress this mutant, which could be reflective of differences in either protein levels or potencies between Tor1p and Tor2p in this assay (our unpublished results). In addition, neither the wild-type *TOR1* nor a rapamycin-resistant allele of *TOR1* (ser$_{1972}$→ile$_{1972}$) when present on a centromeric plasmid is able to rescue the *dna2–20* or *dna2–21* mutants (our unpublished results).

The specificity of this rescue was addressed by testing whether other yeast proteins that contain a lipid kinase domain could suppress the *dna2–20* mutant. As shown in Figure 2, Vps34p (a PI 3-kinase involved in vacuolar protein sorting), Stt4p (a PI 4-kinase involved in cell wall integrity), and Pik1p (a nuclear PI 4-kinase) were all unable to rescue the *dna2–20* mutant on YPD when present on multicopy plasmids. A *GAL1*p→*MEC1* plasmid was also unable to rescue the *dna2–20* mutant on galactose, suggesting that other PIK-related family members do not have this activity (our unpublished results).

Regions of Tor1p Essential for Rescue of dna2 *Mutants*

A deletion analysis of Tor1p was conducted to determine whether the rescuing function could be localized within the protein. As is shown in Figure 3, a 99-amino acid N-terminal deletion ablates much of the rescuing activity, but the residual function is removed only by N-terminal truncations that delete the FKBP-12-rapamycin binding (FRB) domain (Δ1–2067 in Figure 3). A 29-amino acid truncation at the C terminus completely

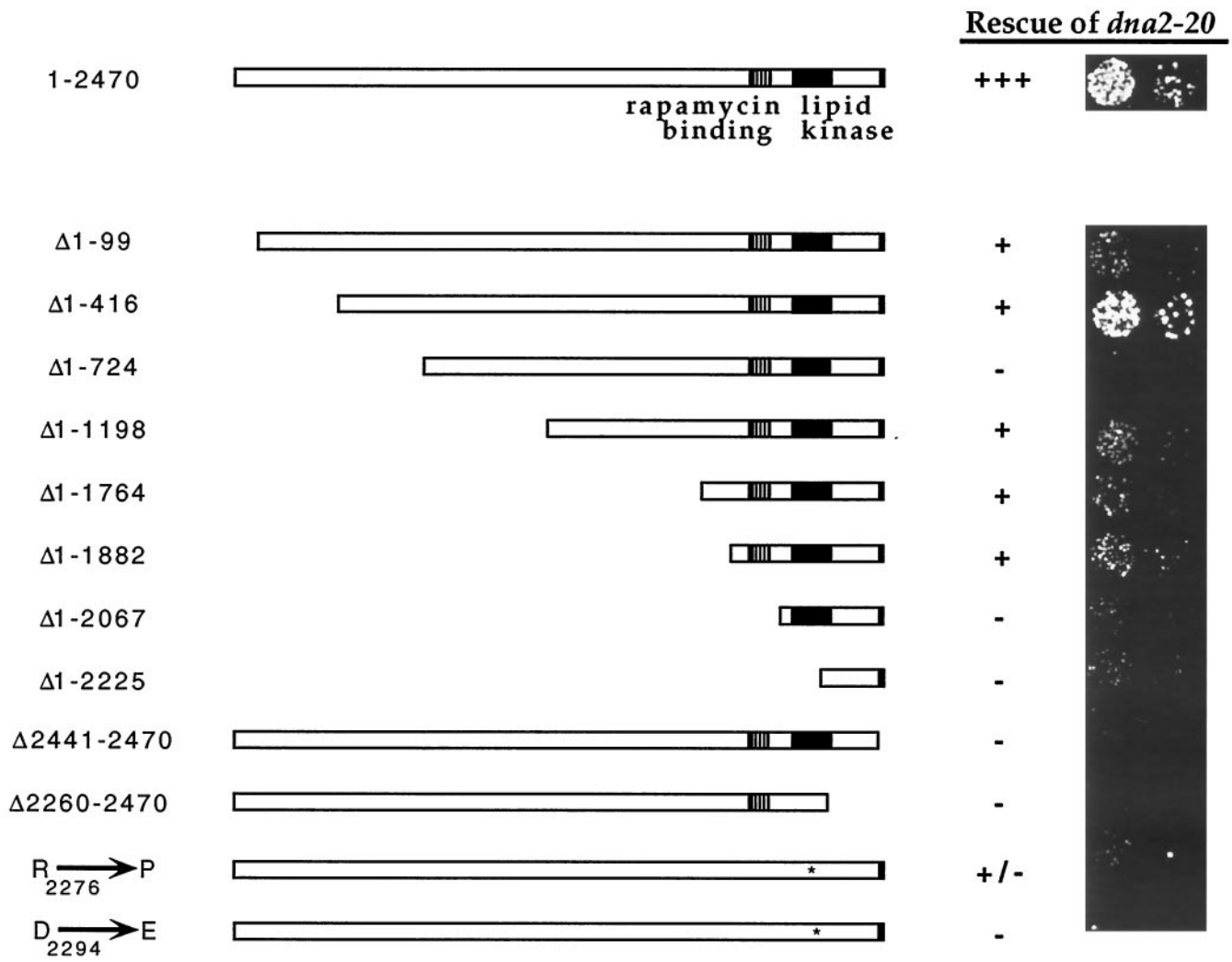

Figure 3. Regions of Tor1p required for rescue of *dna2–20* mutant. The *dna2–20* mutant carrying pYDF88 (*GAL1*p→>*TOR1*) was transformed with various Tor1p deletion constructs (see MATERIALS AND METHODS). Cells were scraped off transformation plates (~100 colonies), resuspended in water, sonicated, and spotted onto YPD plates. Spots represent 1200 and 150 cells applied. Plates were incubated at 30°C for 3 d. The apparently elevated activity seen in the Δ1–416 mutant is not reproducible and is thus represented as +. The absence of suppression by the Δ1–724 construct may be due to low protein expression, as this mutant protein is not detected by immunoblot analysis (our unpublished results).

abolishes rescuing activity and is of interest in that it removes a highly conserved domain that is found in the TOR/FRAP proteins as well as other PIK-related family members (Keith and Schreiber, 1995). The issue of whether the putative kinase activity is required for the suppression was addressed by employing the use of two kinase domain mutants, $arg_{2276}\rightarrow pro_{2276}$ and $asp_{2294}\rightarrow glu_{2294}$, which have been shown to be inactive with regards to their cell cycle function (Zheng *et al.*, 1995). As shown in Figure 3, these mutants are virtually devoid of rescuing activity, although the $arg_{2276}\rightarrow pro_{2276}$ is slightly active. Thus, reminiscent of their roles in G1 progression, both the amino- and carboxyl-terminal domains as well as the kinase activity of Tor1p are required for suppression of the *dna2–20* mutant (Helliwell *et al.*, 1994; Zheng *et al.*, 1995).

dna2 *Mutants Are Compromised by a* tor1 *Deletion*

Since they are rescued by elevated TOR activity, we next tested whether *dna2* mutants are uniquely susceptible to a diminution in TOR function. To do this, we first needed to find conditions whereby the *dna2* mutants are viable with wild-type levels of Tor1p. Both the *dna2–20* and *dna2–21* mutants are rescued for growth on YPD by osmotic stabilizers (e.g., 1 M sorbitol, 0.5 M NaCl) or growth at temperatures above

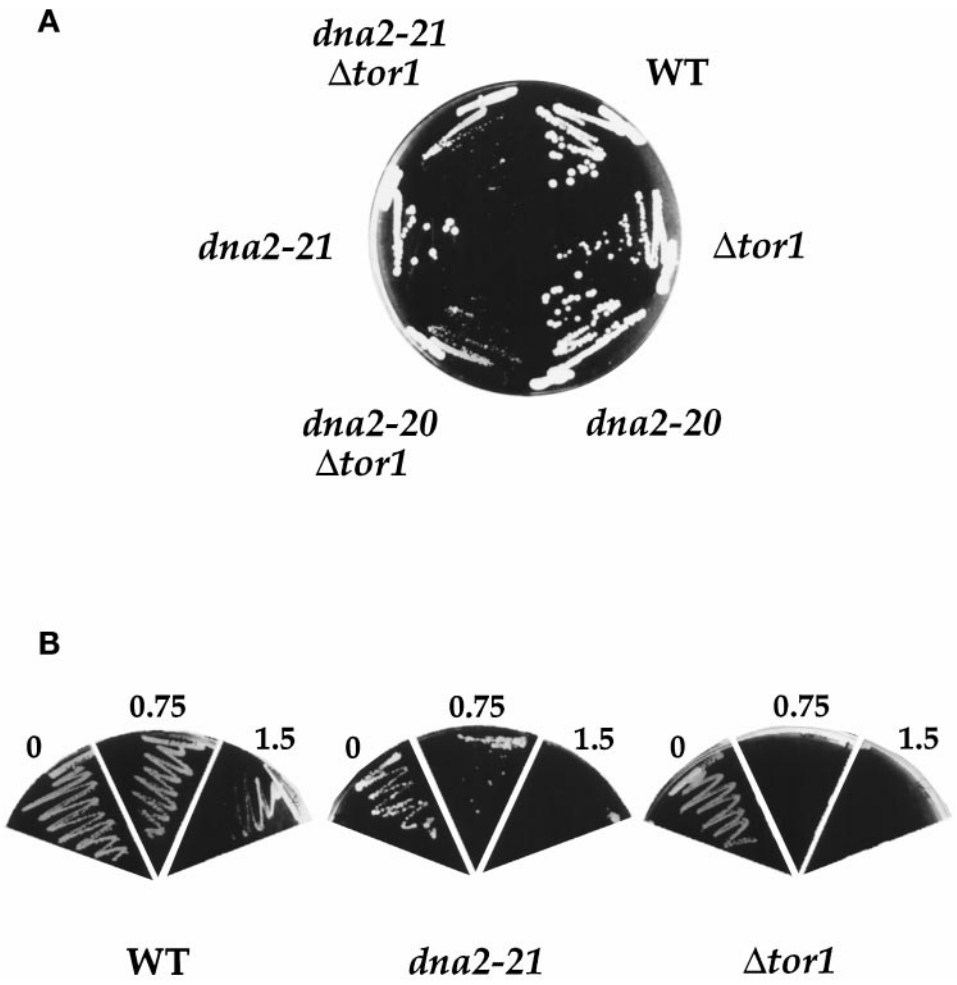

Figure 4. *dna2* mutants show synthetic interactions with the TOR pathway. (A). *dna2 tor1* mutants are slow growing. Strains YMW1 (WT), YDF20 (Δ*tor1), 2–71 (dna2–20*), 2–174 (*dna2–21*), YDF117 (*dna2–20* Δ*tor1),* and YDF121 *(dna2–21* Δ*tor1)* were streaked on YPD plates containing 0.5 M sorbitol at 37°C for 60 h before photographing. (B) *dna2* mutants show increased rapamycin sensitivity. Strains YMW1 (WT) and YDF20 (Δ*tor1), and 2–174 (dna2–21*) were streaked on YPD plates containing indicated amounts of rapamycin at 30°C and photographed after 72 h of growth.

32°C (Figure 4A; our unpublished observations). Using these conditions, we first examined the effect of a *tor1* deletion on the viability of *dna2* mutants. It should be noted that *tor1* cells grow slightly more slowly than wild-type cells at 30°C but are inviable at 37°C in our strain background unless grown on plates containing 0.5–1.0 M sorbitol (our unpublished observations). As seen in Figure 4A, both the *dna2–20* and *dna2–21* mutants form colonies on plates containing 0.5 M sorbitol at 37°C but grow very slowly when combined with a *tor1* deletion even under conditions where the *tor1* strain grows reasonably well. In addition, the *dna2–21* mutant is synthetically lethal with a *tor1* deletion at 32–35°C on YPD (our unpublished observations).

We also tested whether *dna2* mutants show increased sensitivity to rapamycin. Since the *dna2–21* mutant grows very slowly on YPD (i.e., in the absence of Tor1p overexpression), we tested this mutant (and the *tor1* deletion for comparison) for sensitivity to the drug on YPD at 30°C. When compared with wild-type, the *dna2–21* mutant is approximately twofold more sensitive to rapamycin, while a *tor1* deletion strain is approximately three- to fourfold more sensitive (Figure 4B; our unpublished observations). In addition, the *dna2–20* and *dna2–21* mutants are 1.5- to twofold more sensitive to rapamycin when grown on YPD at 37°C (our unpublished observations).

Phenotype of dna2 *Deletion Mutants*

The *DNA2* gene was disrupted by homologous recombination (see MATERIALS AND METHODS). *DNA2*$^+$/*dna2*-Δ*20* cells are viable and show no growth defects at 24°, 30°, or 37°C (our unpublished observations). Upon sporulation and tetrad dissection, these diploids gave rise to only two viable cells in each tetrad (Figure 5A), consistent with the previous observation that *DNA2* is an essential gene (Budd and Campbell, 1995). Upon microscopic examination of the *dna2*-Δ*20* cells that did not form colonies (15 tetrads examined), there were an average of 11 cells (ranging from 1 to 27) generated by each spore with >97% of the cells having a large budded morphology (our unpublished observations). Because the spores

Figure 5. A *dna2-Δ20* deletion mutant is inviable and not suppressed by Tor1p. YDF27 (*dna2-Δ20/DNA2*$^+$) (A), or YDF27 transformed with pYDF113 (*DNA2*) (B), or pYDF77 (*GAL1*p→*TOR1*) (C) were sporulated and tetrads dissected on either YPD (A), (B), or YPGal (C), and plates were incubated at 30°C for 4 d and photographed.

were able to divide, this demonstrates that the *DNA2* gene is essential for viability (and not simply germination) and also indicates that the cell contains a relative excess of functional Dna2p protein. Next, *DNA2*$^+$*/dna2-Δ20* diploids were transformed with plasmids containing either the *DNA2* gene or the *GAL1*p→*TOR1* cassette and tetrads were dissected. Although the *dna2* disruptants are rescued by a Dna2p expression plasmid (Figure 5B), none are rescued by the Tor1p overexpression plasmid (Figure 5C). *dna2* deletion mutants are also not rescued by elevated temperatures or 1 M sorbitol (our unpublished observations).

Dna2p and Tor1p Do Not Physically Interact

One explanation for the observation that the Tor1p-dependent suppression of *dna2* mutants requires the Dna2p protein (Figure 5C) is that Tor1p and Dna2p physically interact. To test this, wild-type yeast was transformed with a plasmid expressing HA-Tor1p under the control of the *GAL1* promoter; this construct is active in *dna2–20*–rescuing activity (our unpublished observations). Yeast extracts were prepared and immunoprecipitated with either anti-Dna2p antibodies or an anti-HA monoclonal antibody. The anti-Dna2p antibodies specifically recognize two polypeptides of Mr 170 and 190 kDa, respectively, a doublet that is consistent with the predicted molecular mass of Dna2p (see MATERIALS AND METHODS). Figure 6A shows that, although the anti-Dna2p antibodies efficiently immunoprecipitate Dna2p, immunoprecipitates with the anti-HA antibody do not show any associated Dna2p. Similarly, although the anti-HA antibody precipitates the HA-Tor1p, none is seen associated with anti-Dna2p immunoprecipitates.

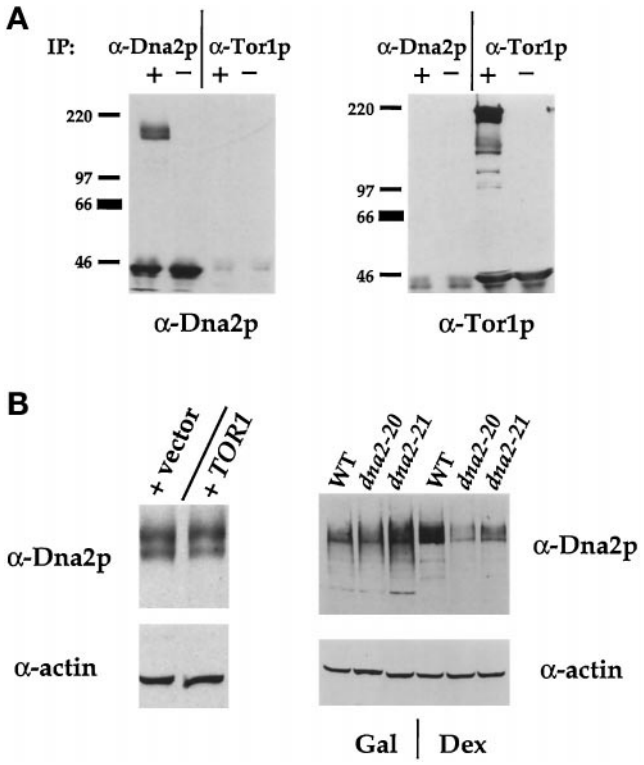

Figure 6. (A) Dna2p and Tor1p do not interact *in vitro.* Extracts from CB018 transformants were prepared and immunoprecipitations (labeled "IP") performed with either affinity-purified anti-Dna2p antibodies (α-Dna2p) or 12CA5 (α-Tor1p) ascites. Washed immunoprecipitates were loaded onto 6% SDS-PAGE gels and immunoblotted with either anti-Dna2p (left) or anti-HA (right) antibodies. + and − indicate the presence and absence, respectively, of extract in the immunoprecipitate. (B). Effect of Tor1p overexpression on Dna2p levels. Left panel, strain CB018 was transformed with either PRS315 (vector) or pYDF125 (*TOR1*), and immunoblots were probed with either affinity-purified anti-Dna2p antibodies or anti-actin antisera. Right panel, strains YMW1(WT), 2–71(*dna2–20*), and 2–174(*dna2–21*) were transformed with pYDF77 (*GAL1*p→*TOR1*) and grown for 5 h in YPGal and either prepared for extracts (Gal) or shifted to YPD for 9 h (Dex) before preparation of extracts. Equal amounts of protein were loaded onto 8% gels and immunoblots probed with either anti-Dna2p (top) or anti-actin (bottom) antibodies. Protein levels were quantified by densitometry and normalized to actin levels.

Effect of Tor1p Overexpression on Dna2p Protein Levels

In view of the role of Tor1p and Tor2p in protein translation, one explanation for our observations is that Tor1p overexpression increases the level of Dna2p within the cell. As shown in Figure 6B (left panel), wild-type yeast transformed with a *GAL1*p→*TOR1* plasmid or a control plasmid contains similar levels of Dna2p when cultured in YPGal, despite equal levels of protein loading as assessed by probing the same blot with anti-hexokinase (Hxk2P) antisera. To test the possibility that Tor1p overexpression specifically stabi-

lizes the *dna2–20* and *dna2–21* mutant alleles, we compared levels of Dna2p in wild-type and *dna2* cells (carrying the *GAL1*p→*TOR1* plasmid) grown in galactose or 9 h after a transfer to glucose-containing media; at this time the mutants contain >80% large budded cells as visualized by microscopy (our unpublished observations). When normalized to actin, the levels of wild-type Dna2p increase by 25% after transfer to YPD while levels of Dna2p from *dna2–20* and *dna2–21* decrease by 38% and 43%, respectively (Figure 6B). Similar results are seen 6 h after transfer to YPD (our unpublished observations).

Phenotype of dna2 *Ts Mutants*

When placed in YPD to repress Tor1p expression, the *dna2–20* and *dna2–21* mutants arrest with a >80% of the cells with a large budded phenotype as assessed microscopically (our unpublished results); the *dna2-Δ1* deletion mutants showed a similar phenotype (see above). Because these results suggest that Dna2p has a role in cell cycle progression, we constructed conditional alleles in which the function of Dna2p could be more easily manipulated. Ten temperature-sensitive (Ts) alleles were generated by *in vitro* mutagenesis of the *DNA2* gene (see MATERIALS AND METHODS). All mutants were recessive with regard to their ability to grow at 37°C, and all arrested with a large budded phenotype within two to three cell cycles after temperature shift (our unpublished results). With regard to their interaction with Tor1p, only two of the mutants showed a slight growth advantage in the presence of Tor1p overexpression, even when tested at semipermissive temperatures; however, this growth advantage was subtle and did not result in the nonsectoring phenotype seen in the original *dna2* mutants (our unpublished results). One of these mutants (*dna2–22*) was selected and integrated into the genome for further study (see MATERIALS AND METHODS). Figure 7 demonstrates that *dna2–22* mutants arrest with a large budded morphology with the nucleus at or near the bud neck. The excess DAPI staining in the mutant is likely due to the increase in mitochondrial DNA despite the cell cycle arrest. In addition, antitubulin staining reveals that these cells have a short mitotic spindle, typical of cells arrested in medial nuclear division before mitosis (our unpublished results) (Hartwell, 1974). Analysis of DNA content by flow cytometry demonstrates that the *dna2–22* mutant arrests with replicated DNA (Figure 7). In addition, *dna2–20* and *dna2–21* mutants, as well as the remaining nine *dna2* Ts mutants, also arrest with replicated DNA (our unpublished results). These data reveal that *dna2* mutants display all of the characteristics of cells arrested at the G2/M border (Pringle and Hartwell, 1981).

dna2 *Mutants Have Defects in DNA Integrity*

Arrest at G2/M is often due to activation of the DNA checkpoint system, a signaling pathway activated by the presence of damaged or incompletely replicated DNA (Hartwell and Weinert, 1989). One way of documenting that *dna2* mutants arrest with damaged or incompletely replicated DNA is to assess their propensity for recombination. Mutants in genes that have a role in DNA replication and/or repair show an increase in recombination and chromosome loss (Hartwell and Smith, 1985). After a brief incubation at 37°C, *dna2–22/dna2–22* diploids have an approximately 10-fold increase in both mitotic recombination and chromosome loss events when compared with wild-type cells (Table 2). As with the defect in growth at 37°C, this phenotype is recessive (Table 2).

We next tested whether the G2/M arrest in *dna2* mutants is dependent upon *RAD9* and *MEC1*, two genes involved in the DNA checkpoint-signaling pathway (Weinert and Hartwell, 1988; Weinert *et al.*, 1994). *Dna2–22 rad9* and *dna2–22 mec1–1* double mutants were generated and compared with wild-type and single mutants with regard to their viability and cell cycle arrest after a brief incubation at either 24°C or 37°C. As a control, we included the *cdc2*, *cdc13*, *cdc9*, and *cdc17* (not shown) mutants, which are known to arrest at G2/M in a checkpoint-dependent manner (Weinert and Hartwell, 1993; Weinert *et al.*, 1994). Table 3 demonstrates that *dna2–22* mutants lose >97% viability after the temperature shift; this loss of viability is largely unchanged in *dna2–22 mec1–1* double mutants and slightly improved in *dna2–22 rad9* double mutants. As expected, the percentage of large budded cells after incubation at the restrictive temperature drops from more than 85% in the *dna2–22* single mutants to 41% and 45% in the *dna2–22 rad9* and *dna2–22 mec1–1* double mutants, respectively. In contrast, although the other *cdc* mutants also lose viability after the temperature shift, a *rad9* mutation exacerbates this effect, consistent with previous results (Weinert and Hartwell, 1993). Of noteworthy exception is *cdc9* (a DNA ligase mutant), which, similar to the *dna2* mutant, displays a fourfold increase in viability after temperature shift if combined with the *rad9* mutation. These experiments demonstrate that the cell cycle arrest seen in *dna2* mutants is due to activation of the DNA checkpoint and suggests that *dna2* mutants sustain some level of DNA damage.

dna2 *Mutants Are Rescued by Checkpoint Mutations*

Because of the paradoxical increase in viability of the Ts *dna2–22 rad9* mutant over the *dna2–22* mutant (see Table 3), we next wished to test whether introduction of checkpoint mutations would also increase the colony- forming ability of *dna2* mutants under semiper-

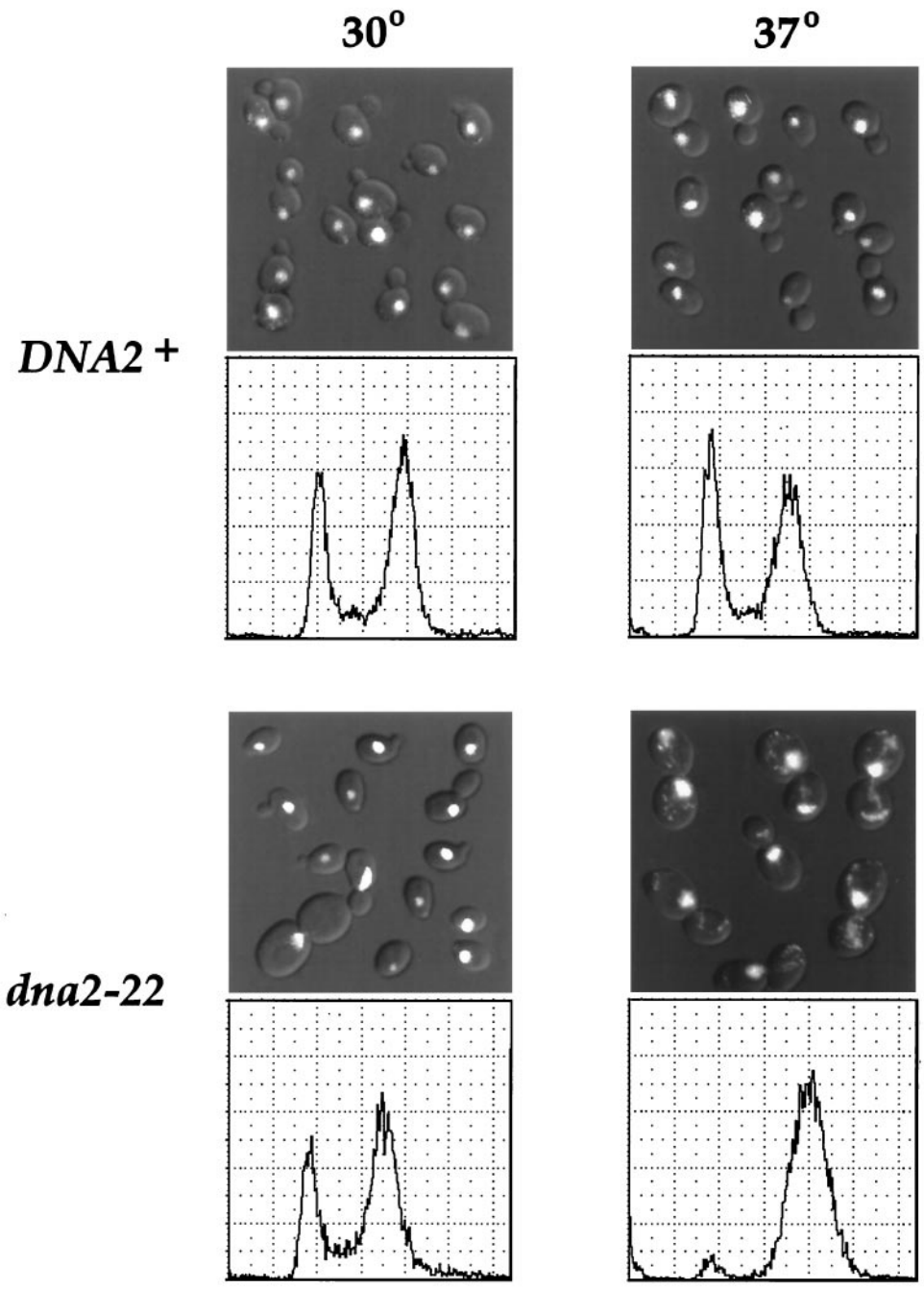

Figure 7. Phenotype of the *dna2–22* Ts mutant. Strains YDF100 (*DNA2*$^+$) and YDF102 (*dna2–22*) were grown at 30°C in YPD to a density of 1.5×10^6 cells/ml, and cultures were split and incubated at either 30°C or 37°C for 4 h. Top panel, DAPI staining. Shown are DIC images with DAPI staining overlaid using Adobe Photoshop 3.0. Bottom panel, cells were processed for propidium iodide (PI) staining and flow cytometric analysis.

missive conditions. Indeed, we find that a *dna2–2 mec1* mutant (but not a *dna2–22 rad9* mutant) forms colonies at 37°C in the presence of osmotic stabilizers while the *dna2–22* mutant is inviable (Figure 8A). As bypass of a checkpoint might explain Tor1-dependent rescue, we next tested whether such mutations might rescue the original *dna2–20* and *dna2–21* mutants by crossing them into our strains and examining the products of meiosis by tetrad analysis. The results for the *dna2–20* mutant are shown in Figure 8B, while similar results were found for the *dna2–21* mutant (our unpublished results). Many tetrads containing >two live cells are seen in both the *dna2 rad9* and *dna2 mec1* crosses, whereas the wild-type cross never produces a tetrad with greater than two viable spores. Although W303 strains (the *dna2* mutants) are being crossed to A364 strains (wild-type and checkpoint mutants) in these experiments (which might account for the low spore

Table 1. Strains used in this study

Strain	Genotype	Reference
YMW1	*MATα ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trp1-1 can1-100 ura3-1*	Zieler et al., 1995
YDF20	YMW1 *tor1-Δ10::TRP1*	This study
CB018	*MAT***a** *Δpep4::HIS3 Δprb1 Δprc1 ura3-52 ade2 trp1 leu2 can1 cir°*	C. Brenner, unpublished
YDF27	YMW1 $\frac{MAT\mathbf{a}\quad dna2\text{-}\Delta 20}{MAT\alpha\quad DNA2^{+}}$	This study
2-71	YMW1 *MAT***a** *dna2-20*	This study
2-174	YMW1 *MAT***a** *dna2-21*	This study
YDF117	YMW1 *MAT***a** *dna2-20 tor1-Δ10::TRP1*	This study
YDF121	YMW1 *MAT***a** *dna2-21 tor1-Δ10::TRP1*	This study
YDF92	*MAT***a** *mec1-1 ura3 trp1 his7*	This study
YDG94	*MAT***a** *mec1-1 ura3 trp1 his7 dna2-22*	This study
YDF96	*MAT***a** *ura3 trp1 his7 leu2 rad9Δ::LEU2*	This study
YDF98	*MAT***a** *ura3 trp1 leu2 rad9Δ::LEU2 dna2-22*	This study
YDF100	*MAT***a** *ura3 trp1 his7 leu2*	This study
YDF102	*MAT***a** *ura3 trp1 his7 leu2 dna2-22*	This study
DBY1034	*MAT***a** *his4-539 lys2-801 ura3-52*	D. Botstein, unpublished
DBY1511	*MATα ura3-52 hom3 leu2 lys2-801 can1-51*	D. Botstein, unpublished
YDF104	$\frac{MAT\mathbf{a}\ his4\text{-}539\ LEU2^{+}}{MAT\alpha\ HIS4^{+}\ leu2}$ $\frac{lys2\text{-}801}{LYS^{+}}$ $\frac{ura3\text{-}52\ CAN1^{+}\ HOM3^{+}}{ura3\text{-}52\ can1\text{-}51\ hom3}$ $\frac{DNA2^{+}}{DNA2^{+}}$	This study
YDF105	$\frac{MAT\mathbf{a}\ his4\text{-}539\ LEU2^{+}}{MAT\alpha\ HIS4^{+}\ leu2}$ $\frac{lys2\text{-}801}{LYS^{+}}$ $\frac{ura3\text{-}52\ CAN1^{+}\ HOM3^{+}}{ura3\text{-}52\ can1\text{-}51\ hom3}$ $\frac{dna2\text{-}22}{DNA2^{+}}$	This study
YDF106	$\frac{MAT\mathbf{a}\ his4\text{-}539\ LEU2^{+}}{MAT\alpha\ HIS4^{+}\ leu2}$ $\frac{lys2\text{-}801}{LYS^{+}}$ $\frac{ura3\text{-}52\ CAN1^{+}\ HOM3^{+}}{ura3\text{-}52\ can1\text{-}51\ hom3}$ $\frac{dna2\text{-}22}{dna2\text{-}22}$	This study

viability), it should be noted that the wild-type and checkpoint strains are strictly isogenic, and thus the observed rescue is due solely to the checkpoint mutations.

Dna2p Executes Its Function during Late S Phase

Since defects in DNA metabolism seem to be the cause for the cell cycle arrest of *dna2* mutants (Tables 2 and 3), we hypothesized that Dna2p might act during S phase. We chose to use loss of viability at 37°C as a marker for when *dna2* mutants pass the Dna2p execution point in the absence of functional protein. Specifically, synchronized cells were subjected to down-

Table 2. Mitotic recombination and chromosome loss in *dna2* mutants

Strain	Temperature	Recomb. ($\times 10^4$)	Monosomes ($\times 10^5$)
DNA2⁺/DNA2⁺	30°C	7.48 ± 1.89	7.04 ± 1.43
	37°C	6.21 ± 0.83	16.3 ± 2.81
DNA2⁺/dna2-22	30°C	7.96 ± 1.45	7.83 ± 0.52
	37°C	6.73 ± 0.38	16.5 ± 3.49
dna2-22/dna2-22	30°C	8.75 ± 2.73	10.92 ± 5.3
	37°C	88.7 ± 16.1	183.2 ± 23.0

Strains YDF104 (*DNA2⁺/DNA2⁺*), YDF105 (*DNA2⁺/dna2-22*) and YDF106 (*dna2-22/dna2-22*) were grown to log phase in YPD to OD = .1 (~3×10^7 cells/ml) and cultured at either 30°C or 37°C for 2 h. This gives ~80% large budded arrest as assayed microscopically and results in a 50% reduction in viability as assessed by comparing microscopic counts with colony counts (our unpublished results). The frequency of mitotic recombinants and monosomic cells was determined as described (see MATERIALS AND METHODS). Numbers represent the averages and SDs from three independent experiments.

Table 3. Effect of checkpoint mutations on *dna2-22* viability and G2/M arrest

	Viability		% Large Budded	
Strain	24°C	37°C	30°C	37°C
WT	97.5 ± .94	96.4 ± 2.3	30.0 ± 5.6	24.0 ± 3.6
mec1	92.3 ± 1.1	77.4 ± 5.8	29.0 ± 2.65	27.3 ± 2.52
rad9	94.6 ± .49	94.8 ± 1.9	25.7 ± 1.53	23.3 ± 2.31
dna2-22	94.6 ± 1.7	2.77 ± 1.6	26.7 ± 3.79	86.3 ± 5.13
dna2-22 mec1-1	89.3 ± 2.0	3.48 ± 1.4	27.3 ± 0.58	4.50 ± 2.65
dna2-22 rad9	93.7 ± 1.3	6.53 ± 2.4	26.3 ± 3.21	40.7 ± 0.58
cdc2	92.7 ± .45	7.7 ± 1.5	ND	ND
cdc2 rad9	43.6 ± .38	.09 ± .02	ND	ND
cdc13	92.7 ± .86	40.7 ± 2.9	ND	ND
cdc13 rad9	70.6 ± 4.0	2.2 ± 1.9	ND	ND
cdc9	91.2 ± 1.1	7.4 ± .50	ND	ND
cdc9 rad9	92.1 ± .66	28.1 ± 2.7	ND	ND

Indicated strains were incubated in YPD at 24° to OD = .02 (approx. 8×10^6 cells/ml). Cultures were split and incubated at 24° or 37° for 4 hr. Cells were sonicated, visualized for large budded morphology, and plated on YPD at 24° for 16 hr before assaying for viability microscopically. Yeast giving rise to colonies containing >30 cells after 16 hr were considered viable. A minimum of 250 colonies was counted for each strain. Numbers represent the averages and standard deviations from 3 independent experiments.

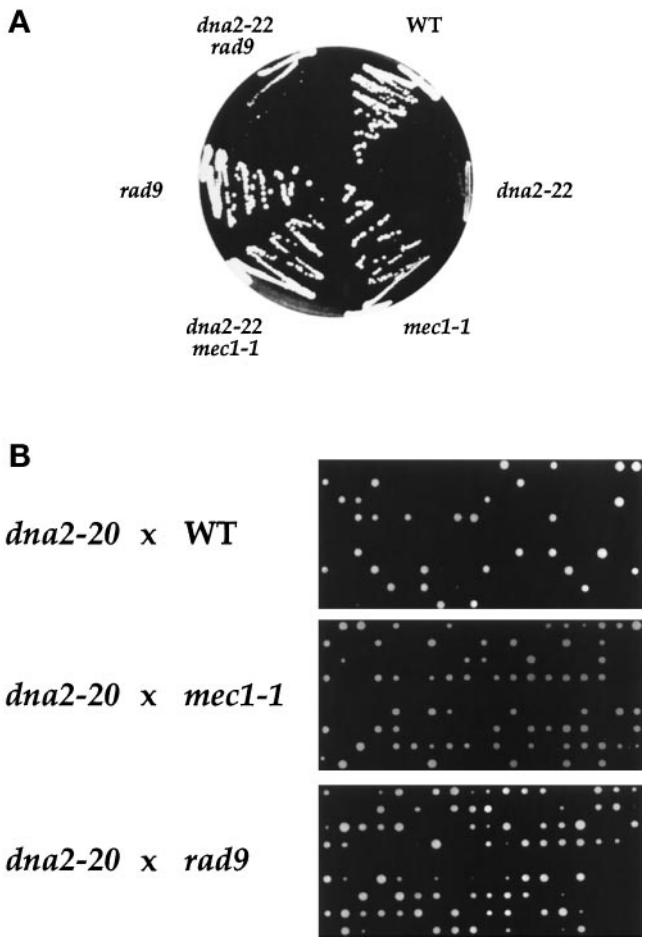

Figure 8. *dna2* mutants are rescued by DNA checkpoint mutations. (A) Strains YDF92 (*mec1–1*), YDF94 (*dna2–22 mec1–1*), YDF96 (*rad9*), YDF98 (*dna2–22 rad9*), YDF100 (WT), and YDF102 (*dna2–22*) were streaked on YPD plates containing 1 M sorbitol at 37°C for 3 d before photographing. (B) Strain 2–71 (*dna2–20*) was mated to strains YDF100 (WT), YDF92 (*mec1–1*), and YDF96 (*rad9*). After sporulation of diploids, tetrads were dissected on YPD. Plates were incubated at 30°C for 3 d before photographing.

shifts in temperature to determine the latest point at which Dna2p can act in the cell cycle. Cells were arrested in G1 with α mating factor at 30°C, released from the arrest at either 30°C or 37°C, and viability was assayed by colony-forming ability at the permissive temperature. Figure 9A shows the results of such an experiment when cells are released from α factor arrest at 30°C. Both wild-type and *dna2–22* cells are effectively synchronized and undergo mitosis (as assessed by both a sudden increase in colony-forming ability as well as by microscopic examination) between 80 and 120 min after release. When cells are released at 37°C, wild-type cells again display a sudden increase in cell number at 80 min (our unpublished results), while the *dna2–22* mutant shows a decrease in colony-forming ability at around this time (Figure 9B). The extent and timing of this effect could be manipulated by including a 37°C preincubation before release from α factor. Prolonged preincubation at 37°C results in a decrease in viability between 20 and 60 min after release, in contrast to the cell death that occurs just after mitosis in cells that have not been preincubated (Figure 9B). That this "late" death truly follows mitosis is supported by the observation that it is prevented by delayed addition of α factor, which allows released cells to enter mitosis but prevents their subsequent entry into S phase (our unpublished results); presumably, these cells are dying during the Dna2p execution point of the following cell cycle. We next wished to determine whether this timing is consistent with an S phase event, and thus the DNA content of the cells was examined at various times after α factor release. Figure 9C demonstrates that, for cells that were incubated for 1 h at 37°C before release, bulk DNA synthesis occurs between 10 and 30 min after release from α factor. The kinetics of DNA synthesis for the cells arrested for 2 h could not accurately be measured due to the accumulation of mitochondrial DNA resulting from the prolonged α factor arrest, which interfered with chromosomal DNA analysis (our unpublished results). These results are consistent with Dna2p having a role in late S phase.

If Dna2p does indeed perform a function during DNA synthesis, then specific inhibition of this event should prevent the loss of viability of *dna2* mutants. HU is an inhibitor of ribonucleotide reductase that arrests cells in early S phase due to a depletion of nucleotide precursors necessary for DNA synthesis (Slater, 1973). Synchronized cells were released at 37° in the presence or absence of HU and then tested for viability 60 min later. Figure 10A shows that wild-type cells are not affected by the addition of HU after α factor release at 37°C. It should be noted that cells are plated before the first mitosis occurs, and therefore the HU-induced arrest does not result in a decrease in colony-forming ability. Most notably, HU effectively restores the viability of the *dna2–22* mutant. Figure 10B shows that, unlike HU, a mitotic inhibitor such as nocodazole is unable to rescue the loss of viability seen in the *dna2–22* mutant. Thus, the Dna2p-dependent step requires DNA synthesis but not mitosis, and, along with the kinetic data presented, likely places the execution point during late S phase.

dna2 *Mutants Replicate the Bulk of Their DNA*

In light of the fact that *DNA2* encodes a putative helicase and that *dna2* mutants have defects during S phase, we wondered whether the gene might encode a protein absolutely required for replication fork progression; specifically, we wished to determine whether Dna2p is required for bulk DNA synthesis. Although the data presented suggest that *dna2* mu-

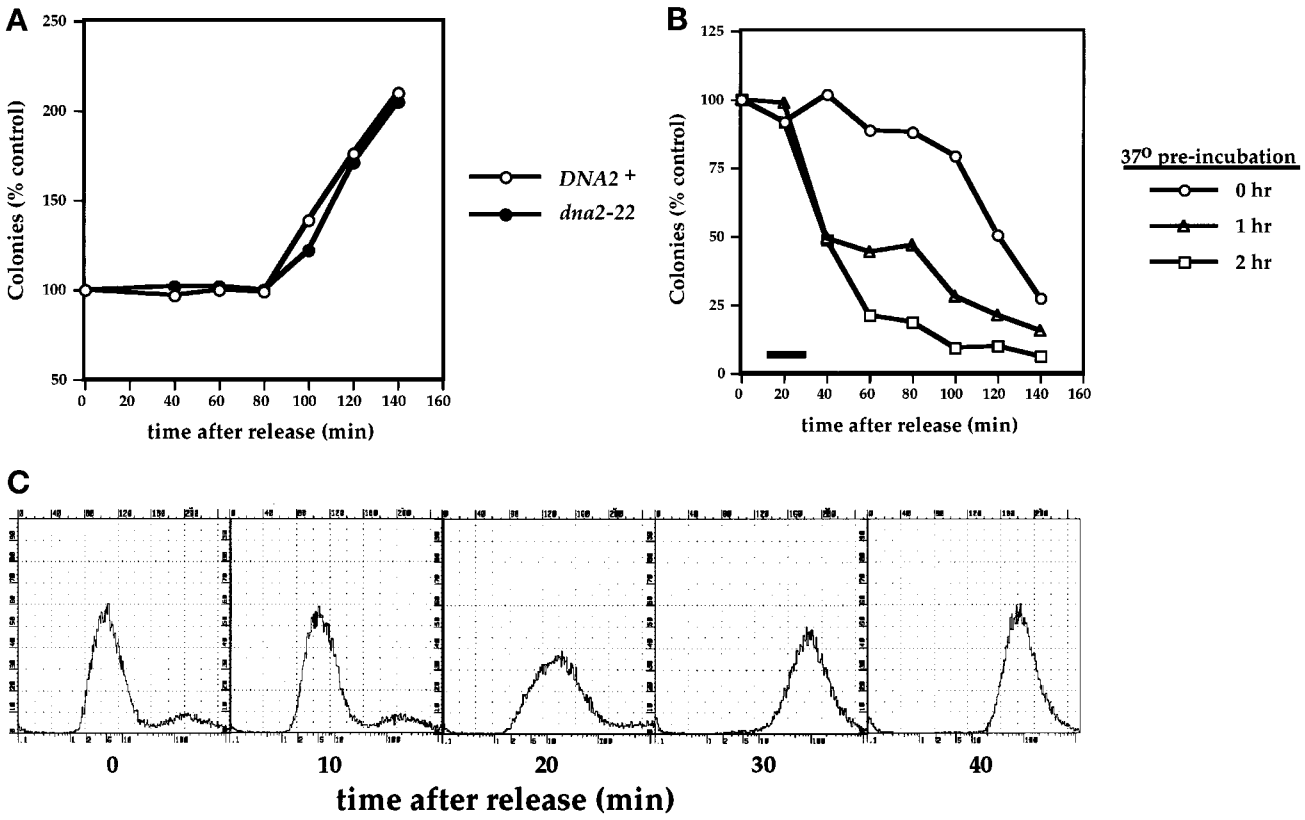

Figure 9. Dna2p executes its function at around the time of late S phase. (A and B) *dna2–22* cells lose viability at 37°C but not 30°C. (A) Strains YDF100 (*DNA2*$^+$) and YDF102 (*dna2–22*) were synchronized with α factor and released at 30°C. In panel B, YDF102 (*dna2–22*) cells were synchronized as in panel A but then preincubated at 37°C for various times before α factor release. Aliquots were taken at the times shown and assayed for colony-forming ability at 30°C. All numbers are normalized to values at t = 0 after α factor release. The black bar in panel B represents the time of DNA synthesis (see panel C) in the 1-h preincubation samples. (C) Kinetics of DNA synthesis in the *dna2–22* mutant. Aliquots of cells from the 1-h preincubation protocol in panel B were taken every 10 min after α factor release and analyzed for DNA content by FACS analysis.

tants are able to replicate their DNA (see Figures 7 and 9C), these experiments might be misleading in light of the fact that it takes several hours at 37°C for the protein in the *dna2–22* mutant to be rendered nonfunctional (Figure 9B); thus, residual Dna2p activity may allow DNA replication in these experiments. We attempted to address this by arresting *dna2* mutants in G1, maintaining this arrest while shifting cells to 37°C for several hours, and then testing for ability of the mutants to replicate their DNA after α factor release. To this end, we included HU in the α factor arrest protocol because HU inhibits the mitochondrial DNA synthesis that interferes with quantitation of chromosomal DNA in cells subjected to a prolonged G1 arrest. The results of such an analysis with both our *dna2–22* strain as well as the isogenic tsDNA2 mutant studied by Budd and Campbell (1995) are shown in Figure 11. The inefficient G1 arrest in the tsDNA2 mutant is likely due to the preponderance of cells in G2 (even in an unsynchronized population) in combination with its extremely slow growth rate (Figure 11). Despite this, both the *dna2–22* mutant as well as the tsDNA2 mutant are similar to wild-type cells with respect to the kinetics and extent of DNA replication. A similar type of experiment was performed using the usual α factor arrest protocol on mitochondrial deficient (ρ°) yeast and yielded similar results (our unpublished results).

DISCUSSION

In a screen to identify novel members of a putative TOR-signaling pathway, we have identified three classes of mutants that require elevated levels of Tor1p for optimal growth. Two of these classes were *tor1* and *tor2* mutants, confirming the ability of this screen to identify genes relevant to our studies. The third class of mutants contained alterations in the *DNA2*, a gene encoding a helicase required for DNA synthesis (Budd and Campbell, 1995). In this report we have attempted both to characterize the nature of the Tor1-dependent

rescue as well as to further clarify the role of Dna2p in the cell.

As predicted, if the rescuing function is indeed related to the redundant G1 progression function, both Tor1p and Tor2p should suppress *dna2* mutants; this is indeed the case (Figure 2). Similarly, as with their role in cell cycle progression, the N terminus as well as the kinase activity of Tor1p are required for efficient rescue of *dna2* mutants (Figure 3). Curiously, unlike *tor* mutants, *dna2* mutants do not show a G1 arrest but, instead, are blocked in G2/M. Most attempts to explain this would include the notion that the functions of Dna2p and Tor1p might be overlapping but not identical. It appears that the activity of Dna2p is not a limiting target of rapamycin treatment, however, because cells transformed with *GAL1*p→*DNA2*, although producing approximately 20-fold higher than physiologic levels of Dna2p, do not acquire any measure of resistance to rapamycin (our unpublished results).

What might be the mechanism of the Tor-dependent rescue? Any hypothesis must take into account the fact that Tor1p does not rescue null mutations in *dna2* (Figure 5C), and therefore complete bypass of Dna2p function is not likely. Because Tor1p cannot rescue any of the more severe Ts *dna2* mutants that we have generated, one possibility is that the effectiveness of the rescue is inversely correlated with the severity of the *dna2* mutation; however, this simple explanation is less likely with the observation that none of the Ts *dna2* mutants are suppressed even at semipermissive temperatures (our unpublished results). This degree of allele specificity is surprising and suggested to us that the two proteins might indeed physically interact, but by both biochemical and two-hybrid analysis we fail to find any evidence for this interaction (Figure 6A; our unpublished results). Aside from physical interaction, allele specificity might occur if Dna2p has multiple functions, only a subset of which are suppressed by the TOR pathway, and only the *dna2–20* and *dna2–21* mutations specifically compromise this function(s). However, we have no evidence that any of our mutants differ with regard to their essential defects.

In light of their G1 progression function (i.e., via effects on translation), one possibility is that the TORs specifically stabilize certain alleles of *dna2*. Although Tor1p overexpression does not affect levels of wild-type Dna2p, we do find that it results in a 40% increase in levels of both the *dna2–20* and *dna2–21* mutant proteins (Figure 6B). However, these data are complicated by the fact that the cells lose viability in the absence of Tor1p overexpression, and thus, even though we use actin as a control, it is possible that Rot1p has an especially short half-life in dying cells. Interestingly, Berg and co-workers (Zieler *et al.*, 1995) have found that mutations in a gene they call *WEB2*

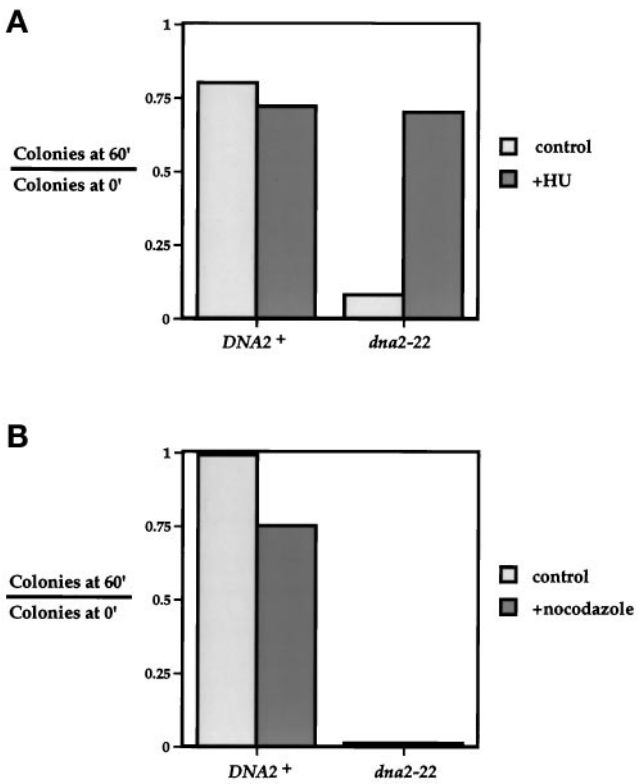

Figure 10. The Dna2p execution step depends upon S phase but not mitosis. (A) *DNA2* executes its function after the HU-dependent step. Strains YDF100 (*DNA2*$^+$) and YDF102 (*dna2–22*) were synchronized with α factor, shifted to 37°C for 3 h, and released from α factor at 37°C into YPD (control) or YPD+0.2 mM HU (+HU). At t = 0 and 60 min after release, cells were diluted into YPD and plated at 30°C. Colonies were counted after 2 d. (B) *DNA2* executes its function independent of the nocodazole-dependent step. Cells were treated as in panel A except that, after α factor release, the strains were incubated in either YPD (control) or YPD+15 μg/ml nocodazole +nocodazole). Cells were then diluted and plated as in panel A. Results are expressed as the number of colonies formed at t = 60 min after α factor release divided by the number of colonies formed at t = 0 min after release.

(which is identical to *DNA2*) are rescued by overexpression of the adenovirus E1A protein . Because E1A is known to elicit most, if not all, of its effects on host cells via changes in transcription, the mechanism of this rescue is probably indirect. One obvious possibility is that E1A induces transcription of *DNA2*, although the authors find that *DNA2* transcript levels are unaffected by E1A overexpression. However, it is conceivable that these studies have revealed that a unique feature of *dna2* mutants is the dramatic phenotypic consequences that can result from very small changes in levels of Dna2p.

However, there is another potential mechanism for the TOR-dependent rescue of *dna2* mutants—namely, bypass of a DNA checkpoint. Our data support the notion that mild defects in *dna2*, although resulting in

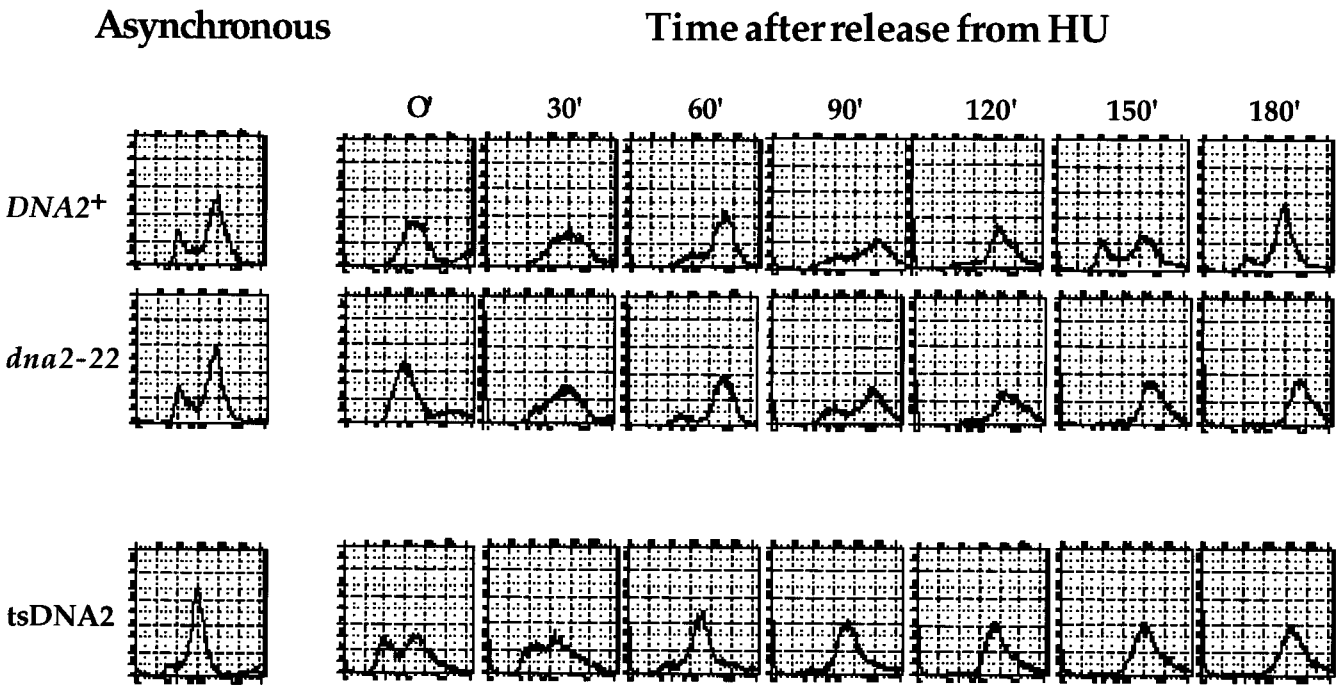

Figure 11. *dna2* mutants replicate the bulk of their DNA. Strains YDF100 (*DNA2*$^+$), YDF102 (*dna2–22*), and tsDNA2 (Budd and Campbell, 1995) were grown to OD 0.25 in YPD and arrested at 30°C with 10 μg/ml α factor. After 3.5 h, cultures were spun down and resuspended in prewarmed (37°C) YPD+0.2 mM HU and incubated at 37°C for 3 h. Cells were then washed and resuspended in prewarmed (37°C) YPD, and 5-ml aliquots were taken and analyzed for DNA content.

a checkpoint-dependent arrest, actually are ameliorated by a defective DNA checkpoint (Figure 8 and Table 3). Indeed, we have direct evidence that both the *dna2–20* and *dna2–21* mutants, those rescued by Tor1p overexpression, are suppressed by introduction of either *rad9* or *mec1* mutations (Figure 8). When overexpressed, perhaps Tor1p and Tor2p, due to their sequence similarity to regions of Mec1p, are able to interfere with Mec1p-dependent functions by binding regulators and/or effectors of Mec1p. This would predict an alteration in checkpoint-dependent activity that would rescue very subtle *dna2* mutants. However, one prediction of this hypothesis is that wild-type cells overproducing Tor1p should be checkpoint deficient, and we find that cells overexpressing Tor1p are no more sensitive to UV or MMS damage than wild-type cells (our unpublished results).

Regardless of the mechanism, a major question is whether Tor1p-dependent rescue of *dna2* mutants is representative of a physiological relationship or whether Tor1p assumes an alternative role in the cell when overexpressed—the latter might be expected to occur after Tor1p mislocalization and/or indiscreet protein-protein interactions. Increased sensitivity of *dna2* mutants to a diminution in TOR activity would support a common biological role for these two proteins. Indeed, we find that compromising TOR function with either a *tor1* deletion or treatment with rapamycin reveals synthetic lethal interactions between *TOR1/2* and *DNA2* (Figure 4).

Notwithstanding its relationship to the TORs, we were initially intrigued that *dna2* mutants not only arrest at G2/M but lose viability. However, it is clear that this is not a unique property of *cdc* mutants that arrest in G2/M because several other mutants of this class lose viability (to a varying degree) after a 4-h arrest (Table 3). Another peculiar observation is that, after a brief incubation at their restrictive temperatures, both *dna2 rad9* and *dna2 mec1* double mutants display similar or even increased viabilities when compared with *dna2* mutants (Table 3). This is unusual for *cdc* mutants of this class, as most mutants with defects in DNA metabolism show at least a 10-fold decrease in viability when combined with checkpoint mutations (Table 3 and Weinert *et al.*, 1994). Interestingly, the only other mutant displaying this paradoxical increase in viability is *cdc9* (DNA ligase). This may suggest that *dna2* and *cdc9* mutants incur a similar type of DNA damage. In the same vein, we find it curious that *dna2* mutants, when grown under semipermissive conditions, are actually rescued by introduction of DNA checkpoint mutations (Figure 8). Again, this is unprecedented for this type of *cdc* mutant, with the exception of the *cdc13* mutation (Weinert

and Hartwell, 1993; Weinert *et al.*, 1994). Our *dna2 mec1* mutants do not acquire lethal amounts of DNA damage, in that they can be restreaked many times, supporting the notion that checkpoint mutations truly rescue the *dna2* mutants and do not simply allow several "suicide" divisions before dying. We are again left with the notion that an intact checkpoint is a liability for *dna2* mutants, especially at semipermissive temperatures. Indeed, there is precedence for DNA checkpoint activation causing undue damage to a cell (Lydall and Weinert, 1995).

We used the loss of viability of *dna2* mutants to determine that Dna2p acts late in S phase, an approach that successfully defined the execution point of topoisomerase II in yeast (Holm *et al.*, 1985). This conclusion is supported by the time of action of the Dna2p (Figure 9) as well as the observation that its function requires ongoing DNA synthesis (Figure 10). Because it has been previously suggested that Dna2p might be required for leading strand synthesis (Budd and Campbell, 1995), we found it curious that *dna2* mutants arrest with a G2 level of DNA (Figure 7). However, one explanation for our observations is that we are not able to completely inactivate the function of Dna2p using our temperature-shift protocol. Indeed, our experiments indicate that it requires approximately 3 h at 37°C to completely abolish Dna2p activity in the *dna2–22* mutant (Figure 9B). However, even using an extended temperature-shift protocol, we still find no difference between *dna2* and wild-type cells with regard to the extent and kinetics of DNA synthesis (Figure 11). Nevertheless, it is still possible that we are unable to incompletely inactivate Dna2p function, and thus mutant cells are synthesizing damaged DNA that is subsequently detected by the DNA checkpoint.

One possible explanation for our data and the observation that the DNA in *dna2* mutants is of low molecular weight (Budd and Campbell, 1995) is that *dna2* mutants are unable to process and/or join Okazaki fragments. Lagging strand synthesis requires that the RNA primer is separated from the DNA template and cleaved from the DNA strand to allow the incoming DNA polymerase to complete synthesis of the upstream fragment. This has been shown to be catalyzed by two proteins, RNaseH1 and FEN1 (also known as MF1/Dnase IV/calf 3′→5′ exonuclease) (Goulian *et al.*, 1990; Turchi *et al.*, 1994). The yeast homolog of FEN1, Rth1p/Rad27p, is essential for viability at 37°C, with mutants arresting at the G2/M border (Reagan *et al.*, 1995; Sommers *et al.*, 1995). Furthermore, it has been shown recently that Dna2p interacts physically and genetically with yeast FEN-1 (Budd and Campbell, 1997). Since FEN-1 is required for Okazaki fragment processing in many in vitro models of lagging strand DNA synthesis, Dna2p might also have a role in the maturation of Okazaki fragments. Further support for this notion may come with an in vitro model of eukaryotic DNA synthesis whose dependence on cellular factors accurately reflects the situation in vivo.

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