MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 2136–2142 Vol. 17, No. 4
0270-7306/97/$04.00+0

A Yeast Replicative Helicase, Dna2 Helicase, Interacts with Yeast FEN-1 Nuclease in Carrying Out Its Essential Function

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Received 25 October 1996/Returned for modification 3 December 1996/Accepted 7 January 1997

We have recently described a new helicase, the Dna2 helicase, that is essential for yeast DNA replication. We now show that the yeast FEN-1 (yFEN-1) nuclease interacts genetically and biochemically with Dna2 helicase. FEN-1 is implicated in DNA replication and repair in yeast, and the mammalian homolog of yFEN-1 (DNase IV, FEN-1, or MF1) participates in Okazaki fragment maturation. Overproduction of yFEN-1, encoded by *RAD27*/*RTH1*, suppresses the temperature-sensitive growth of *dna2-1* mutants. Overproduction of Dna2 suppresses the *rad27*/*rth1*Δ temperature-sensitive growth defect. *dna2-1 rad27*/*rth1*Δ double mutants are inviable, indicating that the mutations are synthetically lethal. The genetic interactions are likely due to direct physical interaction between the two proteins, since both epitope-tagged yFEN-1 and endogenous yFEN-1 coimmunopurify with tagged Dna2. The simplest interpretation of these data is that one of the roles of Dna2 helicase is associated with processing of Okazaki fragments.

In *Saccharomyces cerevisiae*, ARS elements have been identified as replication origins, and these sequences bind ORC, a complex of initiator proteins required for DNA replication (4). The function of initiator proteins after origin recognition and binding, as deduced from studies of prokaryotic replicons, is to recruit a DNA helicase to the origin, which, in collaboration with a single-stranded DNA binding protein, results in unwinding of the origin. The DNA helicase-origin complex in turn recruits proteins involved in DNA elongation: DNA primase, DNA polymerase, and their accessory proteins. For eukaryotes, how origin-binding proteins recruit helicase to the origin and how helicases recruit replication proteins such as DNA polymerases α, δ, ε; DNA primase; PCNA; RF-C; and FEN-1 (8, 9, 28, 29) are not yet known. The first helicase required for chromosomal replication was described only recently, for yeast (2–4). It is called the Dna2 helicase, and while it is a candidate for being involved in initiation, the precise role of the new helicase has not yet been determined.

An *S. cerevisiae* mutant, the *dna2-1* mutant, is inviable at temperatures above 30°C and cannot synthesize high-molecular-weight DNA at the nonpermissive temperature, and it thus appears to be defective in DNA replication (2, 3). Dna2 performs an essential function, since disruption of the gene is lethal. Dna2 is a 170-kDa protein which has five motifs characteristic of DNA helicases in the C-terminal third of the protein, and it is similar to the products of human open reading frame (ORF) HA3331 and a *Caenorhabditis elegans* ORF throughout their entire lengths. The Dna2 protein has DNA-dependent ATPase and DNA helicase activities (2). Both activities are abolished by a K-to-E mutation at amino acid 1080 in the conserved ATP binding motif, GKT (3). This mutant is inviable, showing that ATP hydrolysis and, presumably, DNA helicase are required for the essential function of Dna2 (3). However, there may be additional activities associated with the Dna2 polypeptide, since the large N-terminal domain is not found in other families of helicases and both deletions and point mutations in the N-terminal domain are lethal.

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The precise role of a protein in a multienzyme process such as DNA replication is often suggested by the proteins with which it interacts. It was therefore relevant that a 5′→3′ exo-endonuclease activity is present in Dna2 immunoprecipitates and in immunoaffinity-purified Dna2 protein samples (2, 3). Mammalian DNase IV (FEN-1 or MF1) (7, 10, 14, 22, 23) and the *S. cerevisiae RAD27*/*RTH1* product (ORF YKL510) (9, 11, 19, 24) are two previously described exo-endonucleases with substrate specificities similar to that of the Dna2-associated nuclease activity. (Despite the fact that DNase IV was the first name given to this nuclease, to avoid confusion and reflect a number of recent publications, we refer to this enzyme as yeast FEN-1 [yFEN-1] and to the yeast gene as *RAD27*/*RTH1* in this paper.) Mammalian FEN-1 is required in vitro for joining Okazaki fragments on the lagging strand in the simian virus 40 (SV40) in vitro replication system reconstituted from purified proteins (10, 28, 29). *S. cerevisiae* strains with a deletion of the *RAD27*/*RTH1* gene are inviable at 37°C and are viable, but slow growing, at 23°C. Thus, it appears that yFEN-1 performs a role in an essential process but that some other activity or pathway can compensate for its absence at lower growth rates or lower temperatures (references 19 and 24 and this work).

Several other phenotypes of *rad27*/*rth1*Δ strains are found in other DNA replication mutants. *rad27*/*rth1*Δ strains are sensitive to methyl methanesulfonate and exhibit high rates of mitotic recombination and chromosomal instability (12, 19, 24). *RAD27*/*RTH1* is also required to maintain the stability of poly(GT) repeats, suggesting a role in mismatch repair (12). The yFEN-1 protein has homology to *S. cerevisiae* Rad2 and human XP-G endonucleases, which are required for UV excision repair. However, *rad27*/*rth1*Δ mutants are not sensitive to UV or X-ray treatment (19, 24). Taken together with the mammalian in vitro replication results, these observations argue strongly that yFEN-1 participates in DNA replication and repair in vivo.

We now show that *DNA2* and *RAD27*/*RTH1* interact genetically. High-copy-number plasmids carrying the *RAD27*/*RTH1* gene suppress the temperature-sensitive growth of *dna2-1* mutants, high-copy-number plasmids carrying *DNA2* suppress *rad27*/*rth1*Δ mutant phenotypes, and *dna2-1 rad27*/*rth1*Δ mutants are inviable.

The genetic interactions are probably due to a direct phys-

ical interaction. Coexpression of epitope-tagged versions of their respective genes allows coimmunoprecipitation of the Dna2 helicase and yFEN-1 nuclease, suggesting that they are stably associated in yeast extracts. The endogenous proteins are also associated. If yFEN-1, like mammalian FEN-1, is required for Okazaki fragment joining, then the physical and genetic interactions between yFEN-1 and Dna2 described in this paper suggest that one of the roles of Dna2 may also be to assist in processing Okazaki fragments.

MATERIALS AND METHODS

Strains. The strains used were 3X1549A (α *dna2-1 trp1-289 his3 ura3-1 gal1 tyr3 can1*), BJ5459 (**a** *ura3-52 trp1 lys2-801 leu2Δ1 his3 Δ200 pep4*::*HIS3 prb1Δ1.6R can1*), BJ5459 *rad27*/*rth1*Δ (**a** *ura3-52 trp1 lys2-108 leu2Δ1 his3Δ200 pep4*::*HIS3 prb1Δ1.6R can1*^{s} *rad27*/*rth1*Δ::*LEU2*), MB1 (*DNA2*/*dna2*Δ::*URA3*) (2, 3), and X4119-15d (α *cdc9-1 trp4 hom2 ade8 his1 lys1 gal2*) (from the Yeast Genetic Stock Center. The *pol1-17* strain used in the test for synthetic lethal mutations has been described elsewhere (1).

Plasmids. pGAL18-Dna2-HA contains the *DNA2* gene with the N-terminal hemagglutinin (HA) tag (at M105) cloned into the *Eco*RI site of pGAL18 (2).

pJDCA6 contains the 1.55-kb *ADH1* promoter cloned between the *Eco*RI and *Bam*HI sites of pJDCEN6 (18). pJDCEN6 contains the *ARS1 TRP1* fragment and the *CEN6* centromere fragment. pJDCG6 contains the 0.6-kb *GAL1,10* fragment inserted into pJDCEN6. These plasmids were obtained from Jurgen Dohmen, California Institute of Technology, Pasadena.

pJDA:RAD27MYC contains the *RAD27*/*RTH1* gene with an N-terminal MYC epitope, EQKLISEEDL, inserted into the *Eco*RI site of pJDCA6.

pJDG:RAD27MYC contains the MYC epitope-tagged *RAD27*/*RTH1* gene inserted into the *Eco*RI site of pJDCG6 and thus has the *RAD27*/*RTH1* gene under control of the *GAL10* promoter.

pJDG:DNA2K has the *HA-DNA2* gene inserted into the *Eco*RI site of pJDCG6 and thus has the *HA-DNA2* gene under control of the *GAL10* promoter.

YEp24-3a contains the *RAD27*/*RTH1* gene on a YEp24-based vector (21).

pRS424GRAD27MYC contains the *RAD27*/*RTH1* gene tagged with MYC under galactose control in the pRS424 vector (5), which has a 2μm origin of replication and a *TRP1* selectable marker and into which a 600-bp fragment containing the *GAL1,10* promoter was inserted between the *Eco*RI and *Bam*HI sites. pRS424GRAD27 contains the untagged *RAD27*/*RTH1* gene.

Cloning and epitope tagging of the *RAD27*/*RTH1* gene. The *RAD27*/*RTH1* gene was present on a clone previously studied in our laboratory, YEp24-3a (11, 21). The oligonucleotides MB37 (5′AAGAACACAATGTACCCATACG ACGTCCCAGACTACGCTAGCTTGGGTGGTCCAATGGGTATTAAAGG TTTGAAT) and MB38 (5′AAAGAATTCTATTACAATGATTTCCAACATG) were used as primers in a PCR with YEp24-3a as a template to create a 1,400-bp product containing an HA epitope-tagged *RAD27*/*RTH1* gene. The PCR fragment was cut with *Eco*RI and inserted into the *Eco*RI site of pJDG6 to create pJDG:RAD27HA. The oligonucleotides MB36 (AAAGAATTCACAATG GAACAAAAGCTTATTTCTGAAGAAGACTTGATGGGTATTAAAGGTT TGAAT) and MB38 were used as primers in a PCR with YEp24-3a as a substrate. The resulting 1,400-bp product contains the *RAD27*/*RTH1* gene with a MYC epitope tag at the N terminus. This PCR fragment was cut with *Eco*RI and inserted into the *Eco*RI sites of pJDG6 and pJDA6 to create the plasmids pJDG:RAD27MYC and pJDA:RAD27MYC, respectively. The tagged *RAD27* genes encode functional proteins, since they complement the growth defect of the *rad27*/*rth1*Δ::*LEU2* mutant at 37°C.

Polyclonal Dna2 antibody. A peptide corresponding to the 11 C-terminal amino acids of Dna2 was used to raise antibody to Dna2 by injection into rabbits.

Disruption of the *RAD27*/*RTH1* gene. For gene disruption, the 4,250-bp *Kpn*I-*Pst*I fragment from YEp24-3a (21) containing the *RAD27*/*RTH1* gene and parts of ORFs YKL505 and YKL513 (11) was inserted into pB/S-SK$^-$ at the *Kpn*I/*Pst*I site, creating pB/S510. The *RAD27*/*RTH1* gene is contained in a 1,590-bp *Hin*dIII fragment on the resulting plasmid. pB/S510 was then cut with *Hin*dIII and religated to create the plasmid pB/S510Δ, effectively deleting the *RAD27*/*RTH1* gene on the plasmid. The oligonucleotides MB48 (5′AAAAAGCTTAAGATG CAAGAGTTCGAATC3′) and MB46 (5′AAAAAGCTTTCATGTTTTCTGTT ACACC3′) were used in a PCR with chromosomal DNA to create a fragment containing the *LEU2* gene, which was then cut with *Hin*dIII. The PCR fragment was cloned into the *Hin*dIII site of pB/S510Δ to create pB/S510::LEU2. To create the deletion of the *RAD27*/*RTH1* gene, the latter plasmid was cut with *Kpn*I and *Pst*I and used to transform BJ5459. The structure of the disruption was analyzed by PCR and Southern blotting, verifying that integration occurred at the *RAD27*/*RTH1* locus. The mutant grew at 23 but not 37°C (see Table 1) and was UV and X-ray resistant (2a). The temperature-sensitive growth was complemented by a functional *RAD27*/*RTH1* gene.

Test of ability of *RAD27*/*RTH1* to suppress a *dna2*Δ mutant. The heterozygous *DNA2*/*dna2*Δ::*URA3* strain MB1 (2) was transformed with either pRS424GRAD27 or pRS424, without an insert. pRS424, which carries the *TRP* gene as a selectable marker, contains a 2μm origin of replication and should segregate evenly to all four spores during meiosis. pRS424GRAD27 carries a galactose-inducible *RAD27*/*RTH1* gene on the high-copy-number pRS424 plasmid. The plasmid-containing strains were sporulated and dissected on glucose and on raffinose-galactose plates. Additional control strains carrying the wild-type *DNA2* gene on the same plasmid showed four viable and no inviable spores on glucose and on raffinose-galactose plates. All spores were Trp$^+$. The strains carrying the vector alone gave two viable (Ura$^-$) and two inviable spores, as previously reported and as expected for an essential gene (3). Results obtained with the *RAD27*/*RTH1* plasmid are given in Results.

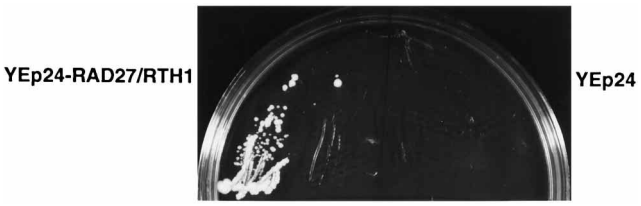

FIG. 1. Suppression of temperature-sensitive growth of the *dna2-1* mutant by high-copy-number plasmids carrying *RAD27*/*RTH1*. Strain 3X1549A cells were transformed with the plasmids YEp24 and YEp24-3a. Colonies were streaked on plates and placed at 37°C.

Protein isolation. Protein was purified as previously described (2). Western blotting was performed as previously described (2).

RESULTS

Genetic interactions between *DNA2* and *RAD27*/*RTH1*. In our previous work, a nuclease activity was observed in immunoprecipitates of extracts of strains expressing HA epitope-tagged Dna2 and in the immunoaffinity-purified HA-Dna2 protein (2, 3). The nuclease was specific for a substrate made by annealing M13 DNA and a 38-mer in which 24 bp was complementary to M13 and 14 bp on the 5′ end was noncomplementary. The nuclease cleaves the oligonucleotide to give a 14- to 16-nucleotide single-stranded product, suggesting that it cleaves zero, one, or two nucleotides from the junction of the single- and double-stranded DNAs. Unlike the Dna2 helicase, the nuclease does not require ATP. Biochemical evidence suggested that the two activities interact, since the product of the nuclease reaction was one or two nucleotides longer in the presence of helicase activity (3). The similarity of the specificities of this nuclease and yFEN-1 nuclease, as well as the fact that both proteins appear to participate in DNA replication, suggested that the nuclease associated with Dna2 helicase might by yFEN-1 (9).

Investigation of the proposal that yFEN-1 interacts with Dna2 helicase. Suppression of temperature-sensitive mutations by high-copy-number plasmids carrying a second gene or by single-copy plasmids expressing elevated levels of a second gene product from a regulated promoter has been used as genetic evidence that two gene products interact in vivo. The plasmid YEp24-3a, previously identified in our lab, is a high-copy-number, YEp24-based plasmid that has an insert from chromosome XI carrying the *RAD27*/*RTH1* locus, which encodes yFEN-1 (6, 11, 20, 21). The *dna2-1* strain was transformed with the recombinant plasmid YEp24-3a. Transformants carrying YEp24-3a grew at 37°C, but transformants with the vector YEp24 did not (Fig. 1).

To ensure that suppression was due to the *RAD27*/*RTH1* gene on the plasmid and not to the other yeast gene present on the YEp24-3a insert, *ABF1*, the *dna2-1* mutant was also transformed with either pJDG:RAD27MYC or pJDA:RAD27MYC, single-copy plasmids expressing only the tagged yFEN-1 protein, under control of the galactose-inducible *GAL10* promoter or the strong constitutive *ADH1* promoter, respectively. pJDG:RAD27MYC transformants grew at 37°C on galactose-con-

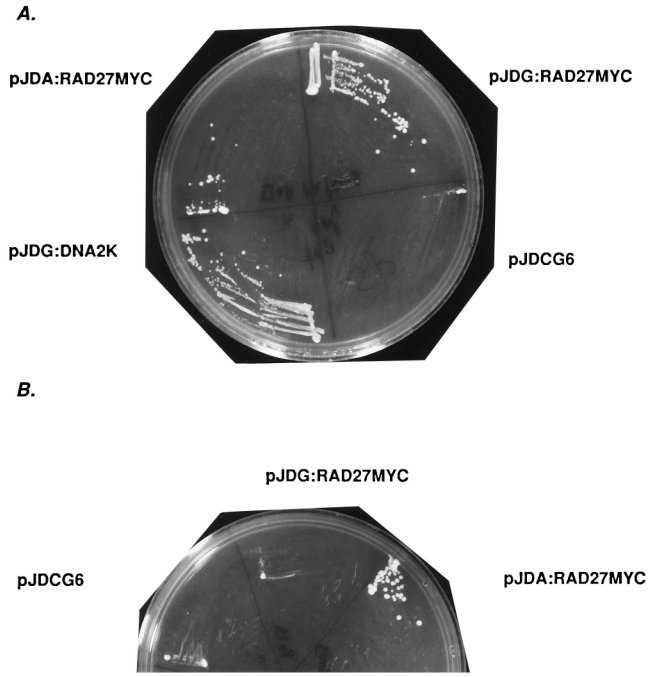

FIG. 2. Suppression of temperature-sensitive growth of the *dna2-1* mutant by high-copy-number plasmids carrying tagged versions of *RAD27*/*RTH1*. 3X1549A cells were transformed with the plasmids containing the indicated gene (see Materials and Methods). DNA2K refers to the wild-type Dna2 protein with lysine in the conserved GKT motif. The *DNA2* gene is expressed from the *GAL10* promoter. The *RAD27*/*RTH1* gene has been expressed with two different plasmids, one containing the *ADH1* promoter and the other containing the *GAL10* promoter. (A) Growth of the transformants on glucose-containing plates; (B) growth on raffinose-galactose-containing plates.

taining plates but not on glucose-containing plates (Fig. 2). Thus, suppression occurred, and suppression required induction. *dna2-1* strains containing pJDA:RAD27MYC grew on all carbon sources. Suppression of *dna2-1* by overexpressed yFEN-1 was not as effective as complementation by a *DNA2* gene, however, since colonies of *dna2-1* cells transformed with yFEN-1-overproducing plasmids and incubated at 37°C contained a significant number of large budded cells and were slower growing than Dna2$^+$ cells.

As previously demonstrated, a strain with a deletion of *DNA2* is inviable (3). Suppression of the *dna2-1* mutant raised the question of whether high-copy-number plasmids carrying *RAD27*/*RTH1* could suppress the *dna2*Δ mutation. Strain MB1, which has the genotype *DNA2*/*dna2*Δ::*URA3*, was transformed with pRS424GRAD27 carrying the *TRP1* gene and a number of control plasmids (see Materials and Methods). The transformants were sporulated and dissected, and spores were allowed to grow at 23°C. Fifteen tetrads derived from the pRS424GRAD27-containing diploid contained two viable and two inviable spores, showing that yFEN-1 overproduction cannot suppress the *dna2*Δ mutation. The viable cells were Trp$^+$ Ura$^-$. Two tetrads, however, contained two large colonies and one small colony, with the latter appearing after 7 to 8 days. The slow-growing colony was Ura$^+$ Trp$^+$ and therefore presumably was a *dna2*Δ strain carrying the *RAD27*/*RTH1*-overproducing plasmid. The slow-growing colonies were inviable at 37°C and arrested with the dumbbell phenotype characteristic of the *dna2-1* mutant at the nonpermissive temperature and of *dna2*Δ spores (2). Thus, while overexpression of *RAD27*/*RTH1* cannot in general bypass a deletion of *DNA2*, some cells that carried the *dna2*Δ allele and the high-copy-number *RAD27*/*RTH1* plasmid could divide. We do not yet understand the origin of this growth.

TABLE 1. Survival of BJ5459 *rad27*/*rth1*Δ transformed with a plasmid overexpressing the *DNA2* gene

Plasmid	Expt	% Viable cells[a] in the presence of:	
		Glucose	Galactose
None	1	0.29	0.18
	2	0.29	0.18
pGAL18-DNA2-HA	1	0.9	14
	2	3.3	14
pRS424GRAD27	1	103	110

[a] Percent viable cells at 37°C compared to 23°C.

Suppression of a *rad27*/*rth1*Δ mutant by overexpression of *DNA2*. We next constructed a haploid strain with a complete deletion of the *RAD27*/*RTH1* coding sequence (see Materials and Methods). As observed previously (19, 24), the *rad27*/*rth1*Δ::*LEU2* strain is viable at 23°C but inviable at 37°C (Table 1). We examined the effect of overexpression of *DNA2* on the growth defect of *rad27*/*rth1*Δ strains at 37°C (Table 1). Strain BJ5459 *rad27*/*rth1*Δ (see Materials and Methods) was transformed with the plasmid pGAL18-DNA2-HA or pRS424 GRAD27, which carries the *TRP1* selectable marker. The transformed strains were purified on raffinose plates and then grown in liquid culture at 23°C. Plating efficiency was then determined, either with or without induction of the plasmid-borne genes. BJ5459 *rad27*/*rth1*Δ transformed with the *RAD27*/*RTH1* gene under control of the *GAL10* promoter grew in the presence of either glucose or galactose. Growth on the noninducing carbon source glucose suggests that yFEN-1 is stable and that very little expression is required for complementation. When the *rad27*/*rth1*Δ strain was transformed with the *DNA2* plasmid, plating efficiency was increased 3- to 10-fold on glucose. However, when the transformants were grown on the inducing carbon source galactose, plating efficiency at 37°C was 100-fold higher than that in strains lacking the plasmid. Complete complementation of *rad27*/*rth1*Δ was observed only with the clone containing the *RAD27*/*RTH1* gene. Thus, overexpression of Dna2 protein can partially compensate for the absence of yFEN-1 at 37°C. The results suggest that Dna2 protein can function in the yFEN-1 pathway or in a parallel pathway.

Synthetic lethality of *dna2*Δ and *rad27*/*rth1*Δ mutations. The temperature sensitivity of the *rad27*/*rth1*Δ strain suggests that another protein can compensate for the essential function of yFEN-1 at 23°C but not at 37°C. The data in Table 1 show that overproduction of Dna2 leads to increased survival of *rad27*/*rth1*Δ strains at 37°C, suggesting that Dna2 may interact with another protein that can perform the same function as yFEN-1 or that Dna2 itself provides an alternative function. If either of these cases applies, then a *dna2-1 rad27*/*rth1*Δ strain might be inviable. A *dna2-1 RAD27*/*RTH1* strain was crossed with a *DNA2 rad27*/*rth1*Δ::*LEU2* strain, and 38 tetrads were dissected. All Leu$^+$ temperature-sensitive segregants could be complemented by *dna2-1* tester strains, and no Leu$^-$ temperature-sensitive segregants could be complemented by *dna2-1* tester strains. Thus, no *dna2-1 rad27*/*rth1*Δ spores were recovered. In the 38 tetrads dissected, the observed ratios of wild-type to *dna2-1* to *rad27*/*rth1*Δ to *rad27*/*rth1*Δ *dna2-1* spores were 33:21:30:0. The probability that these spores would be

recovered at a ratio of 1:1:1:1 is less than 0.001 by chi-square analysis. Furthermore, double mutants were observed at the expected one-in-four frequency when the *dna2-1* strain was crossed with the *rad27*/*rth1*Δ strain carrying a functional *RAD27*/*RTH1* gene on a plasmid (6a). These results suggest that yFEN-1 protein is required in the absence of active Dna2 helicase. In order to show that the absence of double mutants is not a general result of combining the *dna2-1* mutation with other replication mutations, the *dna2-1* strain was crossed with strains carrying either *cdc9-1* (DNA ligase 1) or *pol1-17* (DNA polymerase α). *dna2-1 cdc9-1* and *dna2-1 pol1-17* spores appeared at the expected one-in-four frequency in the crosses between strains carrying the respective mutations. The maximum permissive temperature for the latter double mutants was the same as that for the single mutants, further suggesting that a combination of two replication defects does not necessarily lead to a greater defect in DNA replication and arguing for the significance of the synthetic lethality between *dna2-1* and *rad27*/*rth1*.

One possible explanation for all of the foregoing genetic observations is that the Dna2 protein may have 5′→3′ nuclease activity in addition to DNA-stimulated ATPase and DNA helicase activities, a possibility currently under investigation. Alternatively, a second 5′→3′ exonuclease, similar to yFEN-1 and capable of carrying out the FEN-1 function at 37°C, may also be capable of associating with Dna2. This interaction may be weakened or abolished in the *dna2-1* mutant, explaining the synthetic lethality.

Evidence for physical interaction. The results of the genetic experiments are consistent with the existence of a specific protein-protein interaction between Dna2 and yFEN-1 but are also consistent with indirect interactions, for instance, function in parallel pathways. To confirm that yFEN-1 and Dna2 helicase physically associate, epitope-tagged forms of the two proteins were coexpressed in yeast and were shown to interact by affinity chromatography. The *RAD27*/*RTH1* gene was tagged with the MYC epitope, EGKLISEEDL, as described in Materials and Methods. The fusion protein is 383 amino acids long and has an approximate molecular mass of 46 kDa. The results in Fig. 3 demonstrate that MYC-Rad27/Rth1 is efficiently expressed in yeast and that the 9E10 anti-MYC monoclonal antibody reacts highly specifically with the fusion protein in extracts of yeast expressing it.

The HA-Dna2 fusion protein described above and the MYC–yFEN-1 protein were then coexpressed in yeast. Extracts from cells carrying both HA-Dna2 and MYC–yFEN-1 plasmids were incubated with beads charged with the 12CA5 anti-HA monoclonal antibody and washed extensively as previously described (2). Protein released from the 12CA5 beads by HA-peptide elution (2) was loaded onto an acrylamide gel, blotted onto nitrocellulose, and probed first with the 9E10 anti-MYC antibody and then with the 12CA5 anti-HA antibody. The composite result is shown in Fig. 4A, lanes 1 and 3. A broad band of 160 kDa/corresponding to Dna2 is present, as well as band of about 46 kDa, corresponding to MYC–yFEN-1. The reason for the difference in intensity of the 160- and 46-kDa bands is discussed below. A mock-treated control in which an extract expressing untagged Dna2 and MYC–yFEN-1 was incubated with the 12CA5 beads is shown in Fig. 4A, lane 2. No 46-kDa protein was bound to the beads and eluted with peptide, showing that HA-Dna2 must be present in order to observe MYC–yFEN-1, leading to the conclusion that they interact rather than simply copurify.

Given the relatively low intensity of the MYC–yFEN-1 band in the complex shown in Fig. 4A, which was prepared by using antibody against Dna2, we wished to additionally demonstrate

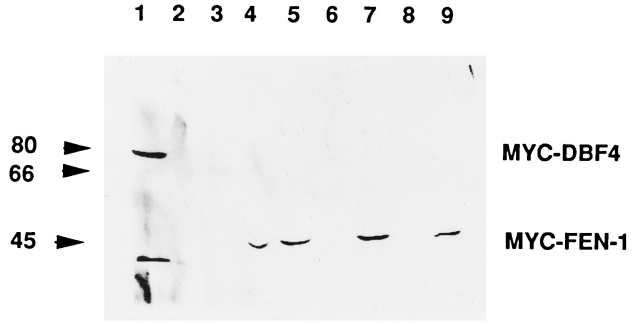

FIG. 3. Western blot demonstrating expression of MYC–yFEN-1. The blot was probed with the anti-MYC antibody 9E10. Lane 1, MYC-DBF4 protein loaded as a positive control for the MYC epitope; lane 2, molecular weight markers; lane 3, extracts from BJ5459 with no expressed MYC–yFEN-1; lane 4, extracts from BJ5459 expressing MYC–yFEN-1 with the *ADH1* promoter and grown in glucose-containing medium; lane 5, extracts from BJ5459 expressing MYC–yFEN-1 under control of the *ADH1* promoter and grown in galactose-containing medium; lane 6, extracts from BJ5459 expressing an HA–yFEN-1 fusion (HA epitope fused to the N terminus of the gene) with the *GAL10* promoter and grown in galactose-containing medium; lane 7, extracts from BJ5459 expressing a MYC–yFEN-1 fusion expressed from the *GAL10* promoter and grown with galactose-containing medium; lane 8, extracts from BJ5459 expressing HA-Dna2 under control of the *GAL10* promoter and grown on galactose; lane 9, extracts from BJ5459 expressing HA-Dna2 under control of the *GAL10* promoter and MYC–yFEN-1 under control of the *ADH1* promoter and grown on galactose. Numbers on the left are molecular weights (in thousands).

that Dna2 coimmunoprecipitates with yFEN-1 from extracts by using an antibody directed against yFEN-1. In order to do this, cells carrying HA-Dna2 and MYC–yFEN-1 were lysed, and immunoprecipitates were prepared by using the 9E10 MYC monoclonal antibody. The immunoprecipitate was then investigated for associated Dna2 by gel electrophoresis and immunoblotting with polyclonal Dna2 antibody. As shown in Fig. 4B, Dna2 is present, along with yFEN-1, in the MYC–yFEN-1 immunoprecipitate. Controls in which extracts of cells carrying either overexpressed or endogenous levels of untagged yFEN-1 (i.e., lacking the MYC epitope) were immunoprecipitated with the MYC antibody showed no Dna2 protein (or yFEN-1) in the immunoprecipitates (Fig. 4B, lanes 2 and 3). (The band present in all three lanes of Fig. 4B is most likely immunoglobulin G.)

In order to confirm that HA-Dna2 interacts with endogenous, untagged yFEN-1, extracts of wild-type or *rad27*/*rth1*Δ mutant cells expressing HA-Dna2 were fractionated through the hydroxyapatite and 12CA5 column steps as previously described (2), loaded onto acrylamide gels, and probed with polyclonal yFEN-1 antibody (kindly provided by Peter M. J. Burgers, Washington University School of Medicine). A band of about 46 kDa, the molecular mass of yFEN-1, is observed in the HA-Dna2 preparation from wild-type cells and is missing from the preparation from a *rad27*/*rth1*Δ mutant (Fig. 5C). The 46-kDa band is most likely yFEN-1 because it reacts with the antibody and because it is present in extracts both of the wild type and of the *rad27*/*rth1*Δ strain carrying a *RAD27*/*RTH1* expression plasmid (Fig. 5A) but is absent from the *rad27*/*rth1*Δ strain without this plasmid (Fig. 5B). Thus, endogenous yFEN-1 copurifies with HA-Dna2. The presence of high levels of endogenous yFEN-1 in the cells expressing HA-Dna2 and MYC–yFEN-1 may lead to competition between endogenous and MYC-tagged yFEN-1 for HA-Dna2 and thus explain the difference between the intensities of the bands observed with the MYC antibody and the HA antibody in the Western blots shown in Fig. 4A.

Further fractionation with Mono Q fast protein liquid chro-

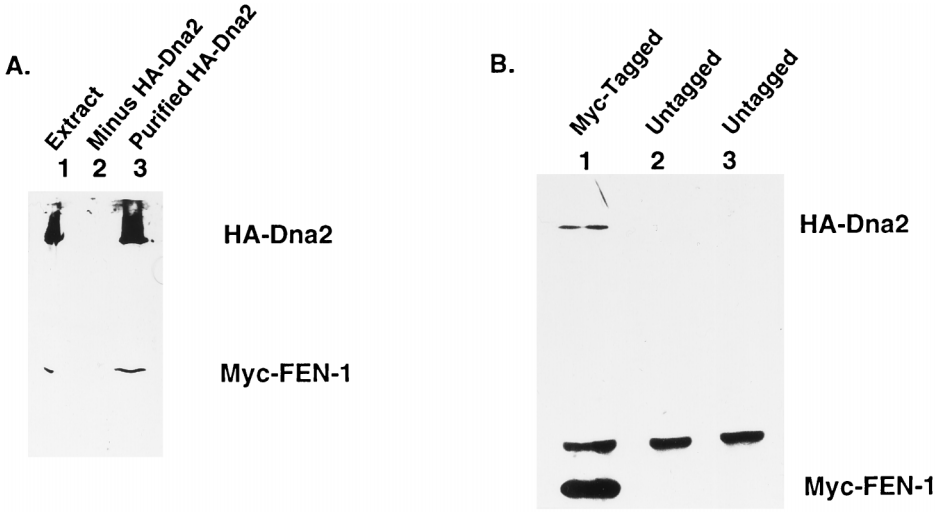

FIG. 4. Physical association of HA-Dna2 and yFEN-1. (A) Protein blot of a 10% sodium dodecyl sulfate gel, probed first with the 9E10 antibody to detect MYC–yFEN-1 and then with the 12CA5 antibody to detect HA-Dna2. Lane 1, blot of extracts from BJ5459 transformed with pGAL18-DNA2-HA and pJDA:RAD27MYC (see Materials and Methods) and grown on galactose-containing medium to demonstrate that coexpression was achieved; lane 2, protein purified through the 12CA5 step from extracts of BJ5459 carrying only pJDA:RAD27MYC (that is, a mock purification control); lane 3, protein from BJ5459 carrying both pGAL18-DNA2-HA (2) and pJDA:RAD27MYC and purified through the 12CA5 step, showing copurification of the tagged proteins. (B) Coimmunoprecipitation of Dna2 with MYC-yFEN1 by using MYC antibody. Cells were lysed with a mortar and pestle in the presence of liquid nitrogen, followed by centrifugation at 29,000 rpm in a Beckman Ti50 rotor. One milligram of lysate was incubated with 10 μg of the antibody 9E10 for 1 h, followed by treatment with 10 μl of 10% protein A for 1 h. Beads were washed with Tris-buffered saline containing 0.1% Tween and boiled, and the lysate was loaded onto a 7.5% polyacrylamide gel. The gel was blotted onto nitrocellulose. The blot was probed first with polyclonal Dna2 antibody at a 1/200 dilution and then with yFEN-1 antibody at a 1/200 dilution. Lane 1, BJ5459 cells transformed with plasmids pGAL18-DNA2-HA and pJDA:RAD27MYC; lane 2, BJ5459 cells transformed with plasmids pGAL18-DNA2-HA and pRS424GRAD27, which contains the *RAD27*/*RTH1* gene without the MYC tag, under control of the *GAL10* promoter; lane 3, BJ5459 cells transformed with plasmid pGAL18-HA-DNA2 alone.

matography failed to separate the two proteins. The apparent tight association of the two proteins suggests that their interaction may be physiologically significant, in accordance with the genetic experiments.

DISCUSSION

We have shown that Dna2 and yFEN-1 interact physically and genetically. First, overexpression of *RAD27*/*RTH1* allows growth of the *dna2-1* strain at 37°C but does not support the growth of a *dna2*Δ strain. Second, overexpression of Dna2 can partially suppress the growth defect of a *rad27*/*rth1*Δ strain at 37°C. Third, *dna2-1 rad27*/*rth1*Δ strains are not observed in a cross of a *dna2-1* strain with a *rad27*/*rth1*Δ strain, suggesting the two mutations are synthetically lethal. Fourth, yFEN-1 binds to immunoaffinity-purified Dna2, when yFEN-1 is expressed either as a MYC epitope-tagged protein or as an endogenous protein. Since FEN-1 has been implicated by several studies in the processing of Okazaki fragments, our results suggest that at least one specific role for Dna2 protein is also maturation of the 5′ ends of Okazaki fragments and that *dna2-1* protein is defective in this processing.

Reconstitution of SV40 DNA replication from purified proteins requires FEN-1 for the processing of Okazaki fragments (10, 28, 29). RNase H is thought to remove the RNA primer up to the last ribonucleotide. FEN-1 is then able to remove the last ribonucleotide. After gap filling by a DNA polymerase, DNA ligase 1 seals the resulting nicks. The proposed role of Dna2 in Okazaki fragment processing leads to a revised molecular model for the processing events. There has been, until now, no direct evidence for a requirement for a helicase, either in the reconstituted SV40 reaction (28, 29) or in a minimal system reconstituted from calf thymus RNase H, FEN-1, and DNA polymerase ε (13, 16, 17, 23, 25, 26). Although SV40 large T antigen is present in the reconstituted SV40 system, all previous studies suggest that it is associated with the leading strand, and there is no model for an additional role on the lagging strand. Furthermore, calf FEN-1 is capable of nick translation when present in the same reaction with DNA polymerase ε (23, 25, 26), and no helicase is required for the reaction to occur. It has also been shown that yFEN-1 interacts physically with PCNA and that PCNA stimulates the exonucleolytic activity of FEN-1 at nicks when RF-C is present (13). Based on these biochemical analyses, it has been proposed that yFEN-1 may interact with polymerase δ or ε through association with PCNA and thus coordinate synthesis and processing of Okazaki fragments. FEN-1 is also active as an endonuclease on an unannealed 5′ region, however (8, 9, 16, 17). In fact, for the yeast enzyme, at physiological salt concentrations and in the absence of PCNA, such a flap structure is the preferred substrate. Several recent studies also suggest that the mammalian enzyme prefers a substrate with a 5′ single-stranded tail (8, 9, 16, 17). Since cellular DNA polymerases do not efficiently catalyze strand displacement, it has been proposed that a 3′-to-5′ helicase also participates in the processing (17), in addition to FEN-1, DNA polymerase, PCNA, and RF-C. Our evidence for physical and genetic interaction between yFEN-1 and Dna2 suggests that Dna2 may perform such a role. Since Dna2 is essential, our results suggest that there is no other helicase that can substitute for Dna2. The 3′-to-5′ directionality of Dna2 helicase translocation is appropriate for the proposed mechanism. In our assays, activation of the helicase through addition of nucleoside triphosphates does lead to a longer product in the nuclease reaction. Although the in vitro reactions described to date for Dna2 suggest that the helicase is most active at a preexisting fork (2), which is not a demonstrated intermediate in Okazaki fragment maturation, this

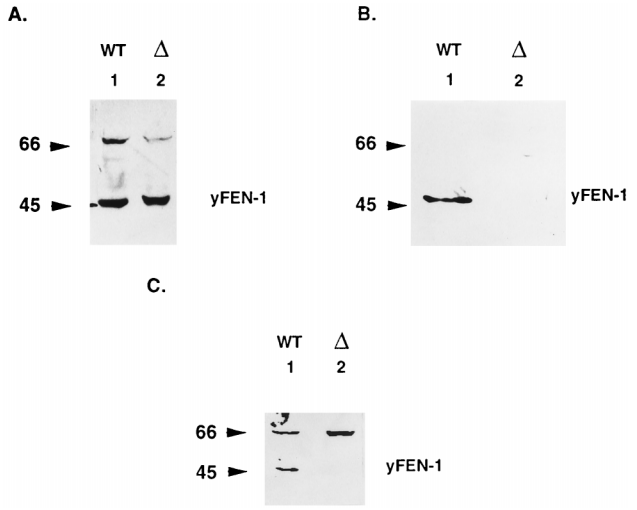

FIG. 5. Physical association of Dna2 and endogenous yFEN-1. Protein (60 μg) from BJ5459 cells transformed with pGAL18-DNA2-HA and purified through the 12CA5 column step was subjected to Western blot analysis with antibody to the yFEN-1 protein (see text). (A) Control showing Western blot of extracts of cells containing overproduced yFEN-1 with the yFEN-1 antibody. Lane 1, BJ5459 carrying pRS424RAD27; lane 2, BJ5459 *rad27*/*rth1*Δ carrying pJDA:RAD27MYC. (B) Control showing Western blot of extracts of BJ5459 (lane 1) and BJ5459 *rad27*/*rth1*Δ (lane 2) with yFEN-1 antibody. Equal amounts of extract were used in each lane. Both strains carried pGAL18-DNA2-HA, as in panel C. (C) Western blot of affinity-purified HA-Dna2. Lane 1, 80 μg of HA-Dna2 protein from BJ5459 transformed with pGAL18-DNA2-HA purified through the 12CA5 step; lane 2, 80 μg of HA-Dna2 protein isolated from BJ5459 *rad27*/*rth1*Δ transformed with pGAL18-DNA2-HA purified through a 12CA5 column. Numbers on the left of each panel indicate molecular weights (in thousands). WT, wild type.

property of the purified nuclease-helicase may be altered in vivo through association with other replication proteins.

rad27/*rth1*Δ mutants are mutators and are defective in mismatch repair reactions that are part of the replication process (12). An attractive function for the nuclease-helicase complex could therefore be a proofreading role for removing errors from the RNA or DNA primers laid down by DNA polymerase α, which lacks a 3′-to-5′ proofreading activity. Since DNA polymerase α is thought to synthesize approximately 20 bases of DNA, such a proofreading activity would be expected to degrade sequences more extensive than just the RNA primer and the first deoxyribonucleotide. Resynthesis with a more accurate DNA polymerase could then take place. Perhaps Dna2 is required to provide a denatured 5′-tailed substrate, leading to more extensive digestion by FEN-1. If this process is not to be too costly, then the extent of degradation must be carefully restricted. What limits degradation remains an open and interesting question, but biochemical characterization of Dna2 may reveal properties that contribute. For instance, if Dna2 operates through the rolling mechanism proposed for *Escherichia coli* Rep helicase (15), then a single cycle of helicase unwinding might be sufficient for the processing event. If so, a helicase containing two subunits that bind ATP but only one that hydrolyzes ATP might still catalyze a single cycle of unwinding. If the nuclease-helicase reaction does perform an error elimination role, then one might rationalize the apparent absence of the requirement for a helicase other than large T antigen in the SV40 processing reaction. Removal of more than one deoxyribonucleotide might be essential to keep error rates low enough to be consistent with viability during chromosomal DNA replication but simply might not be necessary for viral replication. It has recently been shown that DNA polymerase ε cross-links to chromosomal sites of DNA synthesis but not to SV40 replication intermediates, and the differential requirement for the helicase might be a second difference between the viral and chromosomal situations (30).

While there is not much doubt that Dna2 and *RAD27*/*RTH1* interact in the same pathway, mechanisms other than those proposed in the previous paragraph are possible, although less likely. For instance, it is possible that the suppression of the *dna2-1* mutation is not due to stabilization of the mutant helicase through interaction with yFEN-1 but is due to an activity of yFEN-1 that can compensate for a defective function in the N-terminal portion of Dna2. This interpretation merely requires that the Dna2 protein is biochemically bifunctional. The fact that the Dna2 protein or an associated nuclease can compensate for the essential function of yFEN-1 when *RAD27*/*RTH1* is completely deleted from yeast could also be explained by an additional function of the N terminus of Dna2. The *dna2-1* mutation is a P-to-S change at amino acid 504 in a region distinct from the C-terminal helicase domain, which appears to begin somewhere around amino acid 1000. The *dna2-1* mutation is in a region of the protein that is conserved in the possible Dna2 human homolog cDNA HUMKIAAJ-1 and the *C. elegans* homolog CEF43G6-2. Presumably the region performs the same function in yeast, mammals, and worms. Since the only activity known to be associated with yFEN-1 is exo-endonuclease, our data suggest either that Dna2 binds another 5′→3′ exo-endonuclease or that Dna2 itself has 5′→3′ exonuclease activity. If Dna2 binds another nuclease, then the *dna2-1* mutation would destroy the binding domain. If Dna2 has an intrinsic 5′→3′ exonuclease activity, the *dna2-1* mutation might inactivate it. If HA-Dna2 protein is immunoaffinity purified from a strain with a deletion of *RAD27*/*RTH1*, 5′→3′ exonuclease and DNA helicase activities are still observed, which is consistent with either explanation (2a). We favor the idea that the N terminus provides protein-protein interaction sites, however. Recent data have shown that the 5′→3′ structure-specific nucleases, such as FEN-1, fall into a family of related sequences (22). Database searches reveal several ORFs in yeast that fall into this family and that might substitute for yFEN-1, including that for Rad2 (9). Furthermore, using the PILEUP program of the Genetics Computer Group package, we have not been able to detect any homology between the N-terminal region of Dna2 and the remaining members of the 5′-to-3′ exo-endonuclease family (22). Further biochemical experiments will be required to decide the issue, but at the moment, problems with expressing the Dna2 gene or portions of it must be overcome.

There is one additional interesting aspect to the yFEN-1–Dna2 interaction. In searching for targets of the G1 cyclin-dependent kinases, Vallen and Cross (27) have observed that mutations of *rad27*/*rth1* are synthetic lethal mutations in a *cln1 cln2 CLN3* strain. The Cln genes encode three G1 cyclins, which are subunits of the yeast cyclin-dependent kinase, Cdc28. The synthetic lethality of *rad27*/*rth1* is suppressed by overexpression of *CLN2*; thus, *rad27*/*rth1* mutants appear to have an extra requirement for Clns. Vallen and Cross have suggested that *CLN1* and *CLN2* directly activate DNA replication independently of activating Clb-associated kinase activity. They identified a second gene that might be part of the *CLN1*/*CLN2*-dependent pathway, since overexpression of this protein rescues the synthetic lethality of *rad27*/*rth1 cln1 cln2 CLN3*. That gene, called *SEL1*, is allelic to *DNA2* (5a). Our independent demonstration that *rad27*/*rth1*Δ and *dna2-1* are synthetically lethal and that Dna2 can suppress the temperature sensitivity of *rad27*/*rth1*Δ is consistent with the proposal of Vallen and Cross that Dna2 can function in a Rad27/Rth1-

independent pathway in DNA replication, and it will be of interest to investigate whether the activities of Dna2 are altered by Cln-dependent phosphorylation. It is important to note that the Cln-dependent pathway does not entirely replace the Rad27/Rth1 pathway, since the suppression of *rad27/rth1 cln1 cln2 CLN3* by Cln2 requires an active *RAD9* function. *RAD9* controls a checkpoint that monitors completion of DNA synthesis, suggesting that completion of replication is deficient in *rad27/rth1*Δ *cln1 cln2 CLN3/CLN2* strains, leaving some type of lesion that signals the *RAD9* checkpoint. This is also consistent with our observation of only partial suppression of *rad27/rth1*Δ by Dna2 overproduction. In addition, it is interesting that overproduction of *CDC9*, which encodes DNA ligase and is required not for DNA synthesis per se but for joining of Okazaki fragments, is also able to suppress the *rad27/rth1 cln1 cln2 CLN3* synthetic lethality. Thus, the interesting findings of Vallen and Cross also support the role in Okazaki fragment processing proposed here for Dna2. The nature of the proposed contribution to discontinuous synthesis and whether Dna2 has additional roles in DNA replication remain open questions.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM25508 and NSF grant 9507352.

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