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The Nuclease Activity of the Yeast Dna2 Protein, Which Is Related to the RecB-like Nucleases, Is Essential *in Vivo**

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Saccharomyces cerevisiae **Dna2 protein is required for DNA replication and repair and is associated with multiple biochemical activities: DNA-dependent ATPase, DNA helicase, and DNA nuclease. To investigate which of these activities is important for the cellular functions of Dna2, we have identified separation of function mutations that selectively inactivate the helicase or nuclease. We describe the effect of six such mutations on ATPase, helicase, and nuclease after purification of the mutant proteins from yeast or baculovirus-infected insect cells. A mutation in the Walker A box in the C-terminal third of the protein affects helicase and ATPase but not nuclease; a mutation in the N-terminal domain (amino acid 504) affects ATPase, helicase, and nuclease. Two mutations in the N-terminal domain abolish nuclease but do not reduce helicase activity (amino acids 657 and 675) and identify the putative nuclease active site. Two mutations immediately adjacent to the proposed nuclease active site (amino acids 640 and 693) impair nuclease activity in the absence of ATP but completely abolish nuclease activity in the presence of ATP. These results suggest that, although the Dna2 helicase and nuclease activities can be independently affected by some mutations, the two activities appear to interact, and the nuclease activity is regulated in a complex manner by ATP. Physiological analysis shows that both ATPase and nuclease are important for the essential function of** ***DNA2*** **in DNA replication and for its role in double-strand break repair. Four of the nuclease mutants are not only loss of function mutations but also exhibit a dominant negative phenotype.**

Yeast *dna2–1* mutants were originally identified in a screen for mutants defective in DNA replication *in vitro* (1) and were then shown to be defective in DNA replication *in vivo* (1, 2). Since that time, additional *dna2* mutants with similar phenotypes have been identified and characterized (3, 4). Fluorescence-activated cell sorting analysis shows that temperature-sensitive *dna2* mutants can synthesize a full 2C DNA content at 37 °C (3).[1] The DNA synthesized is highly fragmented, however, indicating that, although there is extensive DNA synthesis, DNA replication is incomplete in some way (2). Strains with *dna2* deletions are inviable, showing Dna2 performs an essential function during DNA replication (2–4). Recently, it has also been demonstrated that *dna2* mutants are defective in repair of x-ray-, bleomycin-, and methylmethane sulfonate-induced DNA damage (4, 5).

Dna2 is a 170-kDa protein with six motifs characteristic of DNA helicases in the C-terminal third of the protein. A schematic diagram of the protein is shown in Fig. 1. Genes homologous to *DNA2* have been identified in *Schizosaccharomyces pombe, Xenopus laevis, Caenorhabditis elegans*, and humans (6–8). Immunoaffinity-purified Dna2 has DNA-dependent ATPase activity, DNA helicase activity that requires a 5′ non-hybridized tail adjacent to the duplex region unwound, and a potent endonuclease activity (2, 9–11). The helicase and ATPase activities are required for the essential function of Dna2 since mutation of the ATP binding motif (K1080E) abolishes both ATPase and helicase and results in a gene that does not complement either a *dna2–1* or a *dna2*Δ mutant (9). Other *dna2* mutations that reduce but do not abolish ATP binding and/or hydrolysis support growth under some conditions, showing that the full helicase activity is not essential for viability, and leading to the suggestion that it is the nuclease activity that is essential (4). A recent analysis suggests that Dna2 falls into the RecB class of helicase/nuclease proteins, with homology to the nuclease localized to a short motif in the N-terminal half of the protein, corresponding to the putative active site of RecB nuclease (see Fig. 1).

Preliminary characterization of the nuclease activity associated with immunoaffinity-purified Dna2 suggested that it was a so-called structure-specific nuclease, in that it removed 5′ single-stranded tails adjacent to a duplex region, but not 3′ single-stranded tails and did not digest duplex DNA (10). This suggested to us that Dna2 might have an intrinsic nuclease and/or that it might copurify with yeast flap endonuclease-1 (FEN-1), which has similar substrate specificity (12, 13). Surprisingly, both interpretations appear to be correct. FEN-1 is a 5′ to 3′ exonuclease that also functions as an endonuclease on a 5′ single-stranded flap structure adjacent to a duplex region, cutting at (or near, depending on the context) the junction between the single- and double-stranded region. The human FEN-1 homologue participates in the maturation of Okazaki fragments synthesized during an *in vitro* SV-40 DNA replication reaction (14–16). Yeast FEN-1 is encoded by the *RAD27* gene (17, 18), and the phenotype of *rad27*Δ mutants suggests, albeit indirectly, that the mutants are defective in Okazaki fragment maturation. For instance, *rad27*Δ mutants are viable at 23 °C but not at 37 °C, cause a high level of mutagenesis, but are not defective in repair (17, 18). *rad27*Δ mutants show an increased frequency of duplications at repeated structures, which might occur due to faulty Okazaki fragment processing (19). *rad27*Δ mutants accumulate expansions of di- and trinucleotide repeat DNA tracts, which could be explained by fold-back of FLAP structures on Okazaki fragments at stalled replication forks (17, 20, 21). We have documented strong ge-

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[1] M. E. Budd, L. M. Hoopes, and J. L. Campbell, unpublished results.

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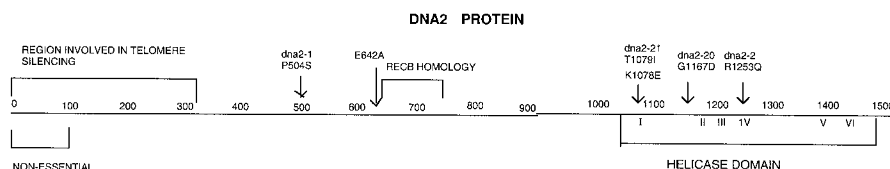

FIG. 1. **Schematic diagram of the yeast *DNA2* gene.**

netic interactions between *DNA2* and *RAD27*. Overexpression of *RAD27* complements *dna2–1* strains and overproduction of *DNA2* suppresses the temperature-sensitive growth defect of *rad27Δ* strains. *rad27Δ dna2–1* strains are inviable. Biochemical studies showed that the affinity-purified Dna2 preparations contained FEN-1 and that FEN-1 and Dna2 co-immunoprecipitated from yeast extracts. This genetic and biochemical evidence led us to propose that Dna2, like FEN-1, functions in Okazaki fragment maturation (10), but left the mechanism ill defined. Dna2 could either substitute for FEN-1, or assist FEN-1 in its function, or repair errors made by FEN-1. Overexpression of *RAD27* also suppresses this sensitivity of *dna2* mutants to x-rays (5), suggesting again an interaction or functional overlap between the two, although *rad27Δ* strains are proficient in DSB[2] repair (17).

We next purified Dna2 from a *rad27Δ* strain, and we found that a potent nuclease activity was still present, suggesting that, in addition to associating with FEN-1, *DNA2* encodes an integral nuclease (10). That Dna2 is itself a nuclease was strongly supported by more extensive characterization of a nuclease associated with recombinant yeast Dna2 protein produced in insect cells (11). These workers found that highly purified Dna2 cleaves single-stranded DNA endonucleolytically, that it prefers 5′ single-stranded tails to 3′ single-stranded tails, and that it has very low endonuclease activity on limited stretches of single-stranded DNA flanked by two regions of duplex (11). Thus, it has the specificity to tailor the 5′ ends of Okazaki fragments during their maturation into continuous DNA strands, providing a biochemical basis for a role in Okazaki fragment maturation. Although several helicase/nucleases are required for DNA repair, Dna2 is the first replication protein that contains both functions.

Herein, we further characterize the Dna2 nuclease/helicase biochemically and genetically. Our results highlight the fact that Dna2 is a vigorous endonuclease, as opposed to a strictly structure-specific nuclease, and that it is regulated by ATP. More important, we show by mutations that eliminate nuclease activity but that leave helicase intact, that the nuclease is integral to Dna2 and that the nuclease domain is essential both for viability of yeast and for repair of x-ray induced damage.

EXPERIMENTAL PROCEDURES

Strains Used—Yeast strains used were: BJ5459 (*MATa ura3–52 trp1 lys2–801 leu2Δ200 his3Δ200 pep4Δ:1–1153 prb1Δ1.6r*) and 4X154–2D (*MATa ura3–52 trp1 his3 leu2*). Strain *dna2–1* for complementation analysis was as described (10). The strain used for the x-ray sensitivity study (Fig. 6*E*) was MB-2–2-5G-6A (*MATa trp1 leu2 ura3 his3 dna2–2::LEU2 sgs1–3::TRP1*).

Oligonucleotides—Oligonucleotides used for construction of mutants and helicase and nuclease assays were: MB55, GGAATGCCAGGGACTGGGGAAACTACTGTTATCGCAGA; MB87, GTTCTTCTGTGGCGTTCCAGGACCACCCAAGCTAGCGTAGTCTGGGACGTCGTATGGGTACATATGGACGATCTCTTCAATTG; MB95, AAATAATACATCGGAATTTAGCACCAACAGGTT; MB94, GAAGCTCTTCTTATTCCCCGGATCCTCAATGGTGATGGTGATGGTGACTTTCATACTCTTGTAGAAT; MB140, TATTGGAGACCAAATGTTCGCTGCCGCTGCAATCACATTGGATATAGA; hpr3, AGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTG; hpr7, AGCTAGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTG; hpr8, AGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTGCCAAGC (44-mer, 30 nt complementary to M13mp18).

Plasmids—The plasmid pB/S:DNA2 has the 6110-bp *Eco*RI fragment containing the *DNA2* gene cloned into the *Eco*RI site of pB/S SK−. The next B/S plasmids were created by site-directed mutagenesis with the above oligonucleotides. The plasmid B/S:87 has the hemagglutinin (HA) epitope YPYDVPDYASLGGP fused to oligonucleotide AA1 and was created by hybridizing pB/S:DNA2 with MB87. In addition an *Eco*RI site was inserted 3 nucleotides upstream of the ATG. The plasmid B/S:87.94 was constructed by hybridizing B/S:87 with MB94, creating a *DNA2* gene with a HA epitope tag at the N terminus and 6-histidine tag at the C terminus. The plasmid B/S:87.94.55 was created by hybridizing MB55 to B/S:87.94, resulting in a K1080E mutation in the ATP binding motif. B/S:87.94.95 was created by hybridizing MB95 to B/S:87.94, resulting in a P504S *dna2–1* mutation. The plasmid B/S:87.94.140 was made by hybridizing MB140 to B/S:87.94, resulting in a DIEE640AAA mutation. The plasmid pGAL:DNA2 was created by cloning the 5.4-kb *Eco*RI fragment from B/S:87.94 into the *Eco*RI site of pGAL18. The plasmid pGAL:Dna2:K1080E was created by cloning the 5.4-kb fragment from B/S:87.94.55 into the *Eco*RI site of pGAL18. The plasmid pGAL:Dna2:E640A was created by cloning the 5.4-kb *Eco*RI site from B/S:87.94.140 into the *Eco*RI site of pGAL18. The pGAL plasmids containing the tagged D657A, E675A, and Y293A mutant Dna2 proteins were created exactly as pGAL:Dna2:E640A. Oligonucleotides used for mutagenesis are available on request. The plasmid pGAL:Dna2:P504S was created by cloning the 5.4-kb *Eco*RI fragment from B/S:87.94.95 into the *Eco*RI site of pGAL18.

The baculovirus expression vector was prepared by inserting the full-length *DNA2* gene in pB/S SK− using the 6-kb *Eco*RI insert from Ycp154–2 containing the *DNA2* gene (2). A *Bam*HI site was engineered at the ATG translation start site of the *DNA2* gene by site-directed mutagenesis with oligonucleotides GATCGTCAGGGGATCCATGCCCGG after purification of U-rich single-stranded phagemid as described in the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad). Full-length *DNA2* was transferred to the baculovirus expression vector pFASTbac HTb (Life Technologies, Inc.), containing a 6xHis tag, using *Bam*HI and *Xho*I to give pbacDNA2. This yields a 6xHis tag at the N terminus of *DNA2*. The plasmid pbacDNA2N, containing the N-terminal 2980 bp of *DNA2* was prepared by deletion of the *Pvu*II-ShoI fragment within the *DNA2* gene. pbacDNA2C was prepared by cloning of the C-terminal 1719 bp of *DNA2*, prepared by polymerase chain reaction, into pFASTbacHTb.

Purification of Dna2 from Baculovirus-injected SF9 Cells—Yeast Dna2 was expressed in insect SF9 cells using the Bac to Bac baculovirus expression system (Life Technologies, Inc.). pFast Dna2 was introduced into *Escherichia coli* DH10BAC and placed on LB agar containing 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracyclin, 100 μg/ml Bluo-gal, and 40 μg/ml isopropyl-1-thio-β-D-galactopyranoside as described in the supplier's manual. White colonies were picked and grown in LB medium containing 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracyclin, and recombinant bacmid DNA was prepared. Bacmids were prepared and introduced into SF9 (or HI5) insect cells using Cell-fectin reagent; recombinant virus were collected and amplified.

Cells were washed with cold Tris-buffered saline buffer and resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% glycerol, and protease inhibitor mix). Cells were lysed by sonication. After cell extracts were cleared by centrifugation at 10,000 rpm at 4 °C, the supernatant was loaded onto a 5-ml heparin-Sepharose column. Dna2-containing fractions were collected with lysis buffer containing 600 mM NaCl after several washes with lysis buffer. The fraction was incubated with Ni^{2+}/NTA-agarose) equilibrated with binding buffer for 2 h at 4 °C. After thorough washing with lysis buffer (100 column volumes), Dna2 was eluted with a gradient from 0 mM to 150 mM imidazole in lysis buffer. The Dna2-containing fraction was dialyzed in 10 volumes of lysis buffer.

The Dna2-containing fraction was loaded onto a 1-ml FPLC Mono Q column. After washing, Dna2 was eluted with a gradient of 100–600 mM NaCl in lysis buffer. Dna2-containing fractions (eluting at 270 mM

[2] The abbreviations used are: DSB, double-strand break; HA, hemagglutinin; 12CA5, anti-HA monoclonal antibody; nt, nucleotide(s); kb, kilobase pair(s); bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; NTA, nitrilotriacetic acid; TBSG, Tris-buffered saline plus glycerol.

NaCl) were pooled and loaded onto a 5-ml 15–40% glycerol gradient and centrifuged at 45,000 rpm in a SW 50.1 rotor for 24 h. Dna2 was identified at all purification steps by DNA-dependent ATPase assay and Western blotting with anti-Dna2 polyclonal antibody. A summary of the purification is shown in Table I.

Purification of Dna2 from Yeast—The pGAL18 plasmids described above were transformed into BJ5459, and cells were grown and extracts were prepared as described previously (2). To purify Dna2 on the Ni^{2+}/NTA-agarose column, the ammonium sulfate precipitated extract was dialyzed in TBSG, pH 8.0, 0.1% Triton X-100 (0.025 M Tris, pH 8.0, 0.15 M NaCl, 10% glycerol, 0.1% Triton X-100). Protein (100 mg) was loaded onto a 1-ml Ni^{2+}/NTA-agarose column. The column was washed with 50 volumes of TBSG, pH 8.0, 0.1% Triton X-100. The column was further washed with 20 mM imidazole in TBSG, pH 8.0, 0.1% Triton X-100. His-tagged Dna2 was eluted with 200 mM imidazole in TBSG, pH 7.6, 0.1% Triton X-100. The protein was concentrated to 1 mg/ml and frozen. Approximately 100-fold purification was obtained.

Dna2 was immunoprecipitated by incubating 5 μg of Ni^{2+}/NTA-agarose-purified Dna2 with 5 μg of 12CA5 antibody for 1.5 h at 4 °C. 10 μl of 10% protein A beads was added followed by a 1-h incubation. Beads were washed five times with TBSG, pH 7.6, 0.1% Triton X-100, 1 mg/ml BSA, then two times with 2× assay buffer. 2× ATPase assay buffer is 20% glycerol, 0.08 M Tris, pH 7.6, 0.01 $MgCl_2$, 0.05 M NaCl, 0.002 M DTT, 1 mg/ml BSA. 2× helicase assay buffer is 20% glycerol, 0.08 M Tris-HCl, pH 7.3, 0.01 M $MgCl_2$, 0.05 M NaCl, 0.002 M DTT, 1 mg/ml BSA. 12CA5 was coupled to CL-4B-agarose beads at a concentration of 10 mg/ml. When 12CA5-coupled beads were used, 5 μg of Ni^{2+}/NTA-purified protein was incubated with 5 μl of 12CA5-coupled beads for 2 h. The protein was then washed as above. When Dna2 was purified from ammonium sulfate precipitates of crude extracts, 0.5 mg of protein was incubated with 10 μg of 12CA5 for 1.5 h, followed by the addition of 10 μl of 10% protein A beads. The substrate was added in a volume of 10 μl and incubated at 37 °C.

Helicase and ATPase Assays—The helicase assay contained 4 mM ATP, 0.01 pM oligonucleotide hpr3 hybridized to M13. 2× helicase assay buffer is 20% glycerol, 0.08 M Tris-HCl, pH 7.3, 0.015 M $MgCl_2$, 0.05 M NaCl, 0.002 M DTT, 8 mM ATP. The standard ATPase assay contained 0.1 mg/ml poly(dA) and 0.2 mM ATP at 25 μM/1 μCi.

Preparation of Nuclease Substrates and Nuclease Assays—For 5′-labeled substrate, oligonucleotides were labeled at the 5′ end with ^{32}P using polynucleotide kinase. Labeled oligonucleotides were hybridized to M13mp18 by heating to 65 °C for 5 min and annealing by cooling to room temperature. Free oligonucleotides were removed by gel filtration using Sepharose CL-4B. For 3′-labeled substrate, the oligonucleotide was annealed to M13mp18 DNA and labeled using Klenow enzyme and [α-^{32}P]dTTP. Unincorporated dTTP was removed by gel filtration.

Purified Dna2 was incubated in a 20-μl reaction (same as ATPase reaction) containing 15 fmol of substrate at 37 °C for 15 min. For denaturing gel analysis, after mixing with sequencing gel loading buffer, samples were boiled and loaded onto a 20% sequencing gel. The gel was run for 90 min at 20 W. Gels were dried and the nuclease products were analyzed with the PhosphorImager (Molecular Dynamics).

RESULTS

Expression of DNA2 in Baculovirus-infected Insect Cells—The *DNA2* gene used in this study contains the full-length *DNA2*, including the 105-amino acid, N-terminal segment absent from the gene used in our previous studies (2). This region does not appear to perform an essential function and shares no homology with *DNA2* genes from other organisms. However, the 105-amino acid region may have a role in transcriptional silencing, since overexpressing this portion of the protein leads to derepression of genes normally silenced at telomeres (22). In evaluating the essential functions of *DNA2*, it seems more prudent to use the full-length protein.

The *DNA2* gene was tagged at the N terminus with 6 histidines, cloned, expressed in SF9 insect cells, and extensively purified as described under "Experimental Procedures" and Table I. In addition to the wild type protein, a K1080E mutant protein, a protein consisting of the 963 N-terminal amino acids (120 kDa), and a protein consisting of the 573 C-terminal amino acids (65 kDa) were expressed and purified. Similar levels of expression and purity were obtained with each of the

TABLE I
Purification of 6xHis-Dna2 from insect cells

	Amount	Volume	Specific activity
	mg	*ml*	*μmol/30 min/mg*
Extract	80	8	
Heparin-agarose	18	10	0.35
Ni^{2+}/NTA	2.8	4	2
Mono Q	0.03	1.5	129
Glycerol gradient	0.01	0.5	160

mutant proteins (Fig. 2). To determine if Dna2, like other helicases, is multimeric, gel filtration analysis was carried out. The Dna2 protein is found primarily as a dimer (Fig. 3). In addition, there is a significant peak of tetramers (Fig. 3) and even some hexamers (not visible in the tracing in Fig. 3). Only the dimeric form of the Dna2 protein shows DNA-dependent ATPase activity, suggesting that the form of the protein active as an ATPase is a dimer (Fig. 3).

DNA-dependent ATPase and Helicase Activity—As shown in Fig. 4*A*, the ATPase activity of the wild type protein requires single-stranded DNA. Either poly(dA) or single-stranded circular M13 DNA is an effective cofactor. Double-stranded DNA fails to stimulate the ATPase. The K1080E mutation abolishes the ATPase activity (Fig. 4*A*), identifying the Walker A box as part of the active site of the DNA-dependent ATPase. The N-terminal 120-kDa protein and the C-terminal 65-kDa protein both lack ATPase (Fig. 4*A*), suggesting that the two domains may interact to activate the ATPase or that the C-terminal fragment does not fold properly, even though it is expressed at levels similar to the wild type (data not shown). The K_m for the ATPase activity was determined from the data in Fig. 4*B* and is 165 μM. Since we have previously shown that 4 mM ATP is required for the DNA helicase activity (2), the low K_m was surprising. Below, we reconcile this discrepancy by showing that much lower concentrations of ATP (100 μM) are required for helicase activity when the nuclease is inactivated by mutation. It is noteworthy that the specific activity of the ATPase associated with yeast Dna2 is almost 100 fold higher than that found in *X. laevis* Dna2 (8), suggesting that there may be some differences between the enzymes from different species.

As reported previously for yeast Dna2 expressed in insect cells and for *Xenopus* Dna2 expressed in insect cells (8, 11), at the levels of protein used here, there was no detectable helicase activity in our preparations on any of the substrates used to assay the activity of the Dna2 protein purified from yeast (for example Fig. 5*A*). The difficulty of detecting helicase in recombinant Dna2 is further addressed under "Discussion" but is not the subject of this paper, which focuses on the nuclease activity.

Nuclease Activity of Dna2—The Dna2 preparations are active as nucleases. To further characterize the substrates and products of the Dna2 nuclease activity, we chose substrates, labeling configurations, and reaction conditions not investigated previously with the recombinant yeast Dna2 protein from insect cells (11). In addition, we compared the wild type Dna2 protein with the K1080E mutant protein. First, we used the helicase substrate, an M13 single-stranded DNA circle hybridized to a 38-nt oligonucleotide with a 14-base noncomplementary 5′ tail (2). The 38-mer was labeled at the 5′ terminus with ^{32}P. The digestion products were analyzed on a native gel. As shown in Fig. 5*A* (*lanes 2*, *4*, *7*, *9*, *11*, and *12*), the major labeled product of the nuclease was 10 nt, with some smaller products, suggesting a preference for cutting this substrate 4 nt away from the junction of duplex and single-stranded DNA (but see below). *Lanes 1* and *6* are controls with protein from mock-infected insect cells carried through the same purification procedure. The K1080E mutant was as active as the wild type

protein (compare *lanes 2* and *4*, *3* and *5*, *7* and *9*, *11* and *12*). When a substrate with an 18-nt 5′ tail was used, the major product was 14 nt (data not shown). These results could suggest that the enzyme monitors distance from the fork. A similar spectrum of products is seen with the enzyme purified from yeast (see Fig. 10).

Since we were concerned that the nuclease might mask the helicase activity by destroying the substrate, we searched for conditions that might inhibit the nuclease preferentially. As shown in Fig. 5*A*, a combination of 2 mM ATP and 2 mM Ca^{2+} completely inhibited the nuclease (*lanes 3* and *5*), yet helicase activity still was not seen. The boiled substrate is shown in *lane 13* to indicate where the product of the helicase would appear. Comparison of *lanes 3* and *5* in Fig. 5*A*, which contain both ATP and Ca^{2+}, to *lanes 8* and *10*, which contain only Ca^{2+}, shows that Ca^{2+} alone was less inhibitory than Ca^{2+} plus ATP. (*Lanes 11* and *12* of Fig. 5*A* are similar to *lanes 3* and *5*, but contain only 1 mM Ca^{2+}.) Comparison of *lane 2* (with ATP) with *lane 7* (no ATP) shows that ATP is inhibitory by itself. Note the increase in smaller products in *lane 7*. We have consistently observed that ATP is inhibitory to the nuclease activity of wild type Dna2 (2, 9), and see below. ATP inhibition is not a result of titration of Mg^{2+}, since 1 mM excess Mg^{2+} was added in the reactions containing ATP and no Ca^{2+}.

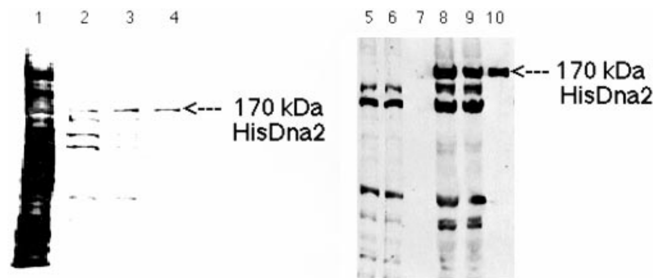

FIG. 2. **Purification of Dna2 from baculovirus-infected insect cells.** 6xHis-Dna2 was purified from insect cells infected by recombinant 6xHis-Dna2 baculovirus as described under "Experimental Procedures" and in Table I. *Lanes 1–4*, silver-stained gel. *Lane 1*, cell extracts; *lane 2*, Ni^{2+}/NTA column eluted fractions; *lane 3*, Mono Q fraction; *lane 4*, glycerol gradient fraction. A mock purification was carried out in parallel with cells infected with virus lacking insert. *Lanes 5–10*, Western blot using Dna2 polyclonal antibody. *Lane 5*, mock cell extracts; *lane* 6, mock Ni^{2+}/NTA column flow-through; *Lane 7*, mock Ni^{2+}/NTA column eluted fraction; *lane 8*, 6xHis-Dna2 cell extracts; *lane* 9, 6xHis-Dna2 Ni^{2+}/NTA column flow-through; *lane 10*, 6xHis-Dna2 Ni^{2+}/NTA column eluted fraction.

In Fig. 5*B*, we show the labeled products of the reaction when Dna2 is incubated with the same configuration of substrate but with the radioactive label at the 3′ terminus of the oligonucleotide, which is in a duplex structure. In this experiment, the duplex region is 30 bp long and the tail is 14 nt. As opposed to the native gel shown in Fig. 5*A*, a denaturing polyacrylamide gel was used in order to detect potential cutting in the duplex portion of the substrate. The major product, as revealed after denaturation, is clearly 30 nt in length, with minor products of 29 and 31 nt. The size of the products labeled at the 3′ end suggests that there is significant cutting at the junction of the single-stranded 5′tail and duplex DNA, that is at the fork. This activity is due to Dna2 protein since it is inhibited by Dna2 antibody, shown in Fig. 5*C*. Taking the results of Fig. 5, *panels A* and *B*, together, we infer that the endonuclease cleaves at the junction, releasing the single-stranded 5′ product, which may then be further degraded. The smaller products are not detectable when label is only at the 3′ end. The 30-nt duplex, by contrast, is stable to further digestion by Dna2, but detectable only when label is at the 3′ end. Comparison of the amount of degradation in Fig. 5 (*A* and *B*) suggests that the enzyme prefers the single-stranded tail to the junction, although it is difficult to quantify the difference in preference. It is clear that Dna2, like FEN-1, can cleave a flap structure. However, Dna2, unlike FEN-1 (13), has a potent activity as a single-stranded endonuclease, yielding products of 10 nt in length and smaller. Thus, the specificities of Dna2 and FEN-1 are overlapping but not identical.

To verify that Dna2 cleaves single-stranded DNA, the 42-nt oligonucleotide, in the absence of M13, was labeled at the 5′ end. After incubation with enzyme, the major products were 10 nucleotides or less (data not shown). Completely single-stranded circular DNA was then used to determine whether the enzyme had any preference for ends, as might be found on immature Okazaki fragments. When M13 circular DNA was used as substrate, it was cleaved to linear, full-length DNA (Fig. 5*D*). Over 60% of the substrate was converted to linear DNA before any fragments smaller than unit length were observed. This is the same pattern of cleavage observed with the RecB nuclease, and suggests that on long DNA molecules Dna2 has no preference for DNA ends over internal sites and that the enzyme is distributive under these assay conditions. This does not rule out that the endonuclease may prefer short tails adja-

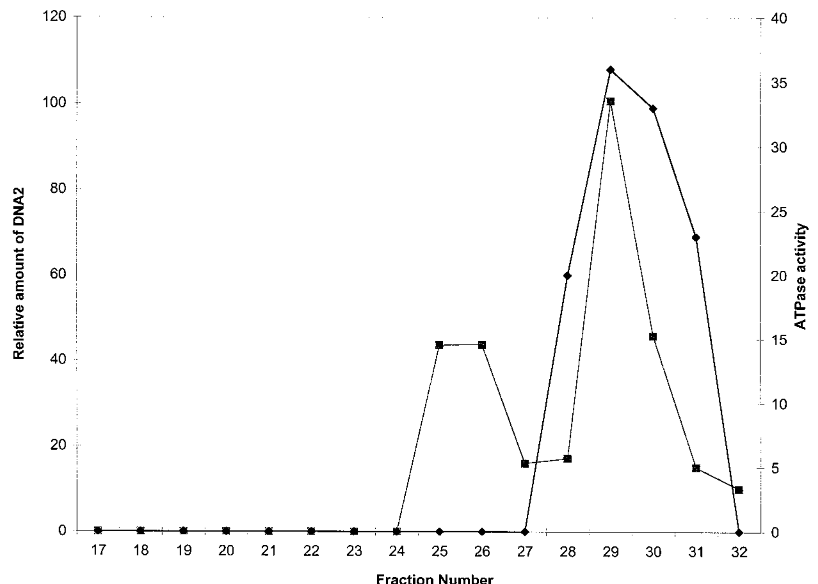

FIG. 3. **Size exclusion analysis of Dna2.** Partially purified Dna2 (0.5 mg, Ni^{2+}/NTA-agarose fraction) was loaded onto an FPLC Superose 6 HR 10/30 column. Fractions (0.5 ml) were collected and analyzed by Western blotting using anti-Dna2 polyclonal antibody. The relative amount of Dna2 in each fraction was measured by densitometer tracing of the Dna2 Western blot. ATPase activity was assayed with 2 μl of each fraction in 20-μl reactions in the presence of 0.2 mM ATP for 30 min. The column was calibrated with thyroglubulin (669 kDa), ferritin (440 kDa), and aldolase (158 kDa). The peaks of these markers were in fractions 25, 29, and 32, respectively. ■, Dna2; ♦, ATPase activity.

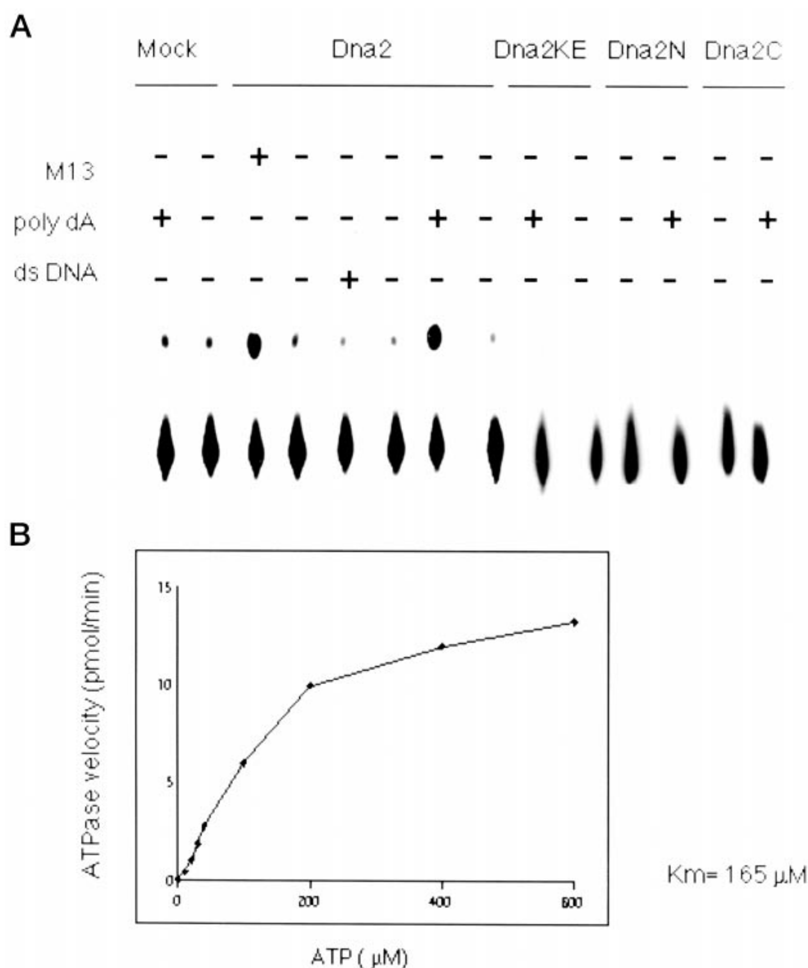

FIG. 4. **ATPase activity of purified Dna2 and mutant proteins expressed in baculovirus-infected insect cells.** *A*, DNA requirement. 6xHis-Dna2 (2 ng) was incubated with various DNA cofactors. *M13*, double-stranded DNA (linearized pRS304) and poly(dA) were each present at 2 μg. ATPase was assayed as described under "Experimental Procedures." *B*, level of ATPase. The assay was carried out as described under "Experimental Procedures" with 2 ng of 6xHis-Dna2.

cent to short single-stranded regions within duplex DNA, where the single-stranded DNA may be too short for efficient binding of Dna2 protein. Other studies suggest that single-stranded tails are preferred to single-stranded regions flanked by duplex DNA when oligonucleotides are used as substrates (11).

Mutations Affecting the Nuclease Activity of Dna2 and Their Effects on Growth of Yeast and Repair of Double-strand Breaks—Two lines of evidence suggest but do not prove that Dna2 encodes the nuclease activity. First, the nuclease activity copurifies with Dna2 from a *rad27*Δ mutant and with Dna2 from insect cells expressing only yeast Dna2 and not yeast FEN-1. Second, the substrate specificity of FEN-1 differs from that of Dna2. To obtain further evidence that the nuclease is intrinsic to Dna2, to examine the location of the nuclease domain in the protein, and to determine if the activity is part of the essential function of Dna2, we have made mutations that target the nuclease. Since at the time this work was begun, we could detect no homology within Dna2 with any known nuclease motifs, our rationale was as follows. The nuclease is probably associated with the N terminus, since helicase occupies the C terminus. Therefore, the first mutation we introduced into the full-length Dna2 protein is the N-terminal *dna2–1* mutation, P504S, originally identified by us (9). This amino acid falls in a region strictly conserved among all Dna2 orthologs. The second mutation was designed to target putative nuclease catalytic residues. Dna2 is a Mg^{2+}-dependent nuclease. Metal catalyzed nuclease reactions often require aspartic and glutamic acids as ligands for Mg^{2+}. In the Exo1 domain of *E. coli* DNA polymerase I, there are two aspartic acid residues in a row and these are conserved in Exo domains from other bacterial and eukaryotic DNA polymerases with 3′ to 5′ exonuclease activity (23). Altering the nuclease activity of Dna2 might involve mutating two or more conserved aspartic or glutamic amino acid residues. The residues DIEE beginning at amino acid 640 are conserved among human, C. *elegans*, and *X. laevis* Dna2 and were therefore deemed candidates for essential catalytic residues. These amino acids were changed to AAAA by site-directed mutagenesis as described under "Experimental Procedures."

Since the time that we constructed the latter mutant, a study has appeared proposing that a region 11 amino acids downstream of amino acid 640 is homologous to the putative nuclease active site of the RecB nuclease family (24). Three additional mutations were then introduced on the basis of the conservation with RecB nuclease. Asp^{657}, Glu^{675}, and Tyr^{693} were changed to alanine. Mutation of the amino acid in RecB equivalent to Dna2 Glu^{675} eliminates the nuclease activity of RecB protein, D1080A (24, 25).

A sixth mutation, K1080E, was introduced into the Walker ATP binding loop. We have already described both the catalytic and functional properties affected by this mutation in the N-terminally 105 amino acid truncated form of Dna2. However, since we had not characterized the full-length Dna2 protein containing this change, the new K1080E mutant serves as an important control (2).

The wild type and six mutant genes, P504S, E640A, D657A, E675A, Y690A, and K1080E, tagged with 6xHis at the C terminus and with the HA epitope at the N terminus, were cloned into the multicopy vector pGAL18 downstream of the galactose-inducible, glucose-repressible yeast *GAL10* promoter. As illustrated in Fig. 6*A*, the *DNA2* gene complemented the *dna2–1*

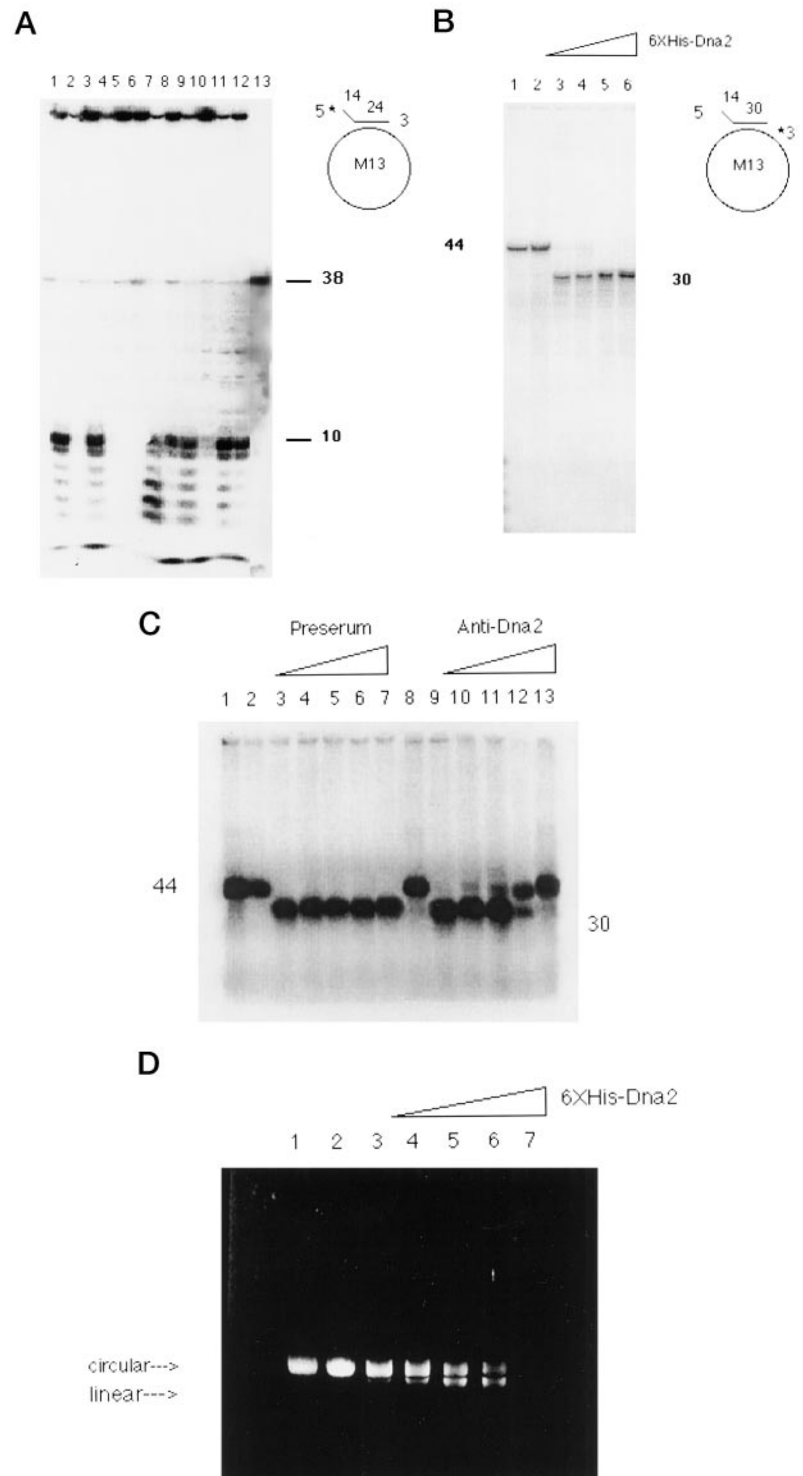

FIG. 5. **Products of endonuclease activity of wild type and mutant Dna2 proteins expressed in baculovirus-infected cells.** Oligonucleotide was annealed to M13mp18 and reactions carried out as described under "Experimental Procedures." Products were analyzed on a neutral gel. *A*, substrate labeled at the 5′ end, 38-nt hpr 3 oligonucleotide. *Lanes 1* and *6*, protein from extract of cells infected with virus lacking the Dna2 gene carried through the affinity purification procedure. *Lanes 2*, *3*, *7*, *8*, and *11* are 6xHis-Dna2 protein. *Lanes 4*, *5*, *9*, *10*, and *12* are 6xHis-Dna2K1080E protein. *Lanes 1–5* test the effect of ATP on the activities of Dna2. They contain 4 mM ATP in addition to the normal reaction mixture. *Lanes 2* and *4* contain ATP only. *Lanes 3* and *5* also contain 2 mM $CaCl_2$in addition to ATP. *Lanes 6–10* test the nuclease in the absence of ATP but in the presence of $CaCl_2$. These reactions contain no ATP, but *lanes 8* and *10* contain 2 mM $CaCl_2$. *Lanes 11* and *12* are similar to *lanes* 3 (6xHis-Dna2) and 5 (6xHis-Dna2K1080E) except they contain only 1 mM $CaCl_2$. *Lane 13* is boiled substrate, no protein. *B*, substrate labeled at the 3′ end, hpr 8 oligonucleotide. Products of reactions were boiled and analyzed on a denaturing acrylamide gel. *Lanes 1* and *2* are boiled substrate and substrate incubated with protein purified from extracts lacking recombinant 6xHis-Dna2 virus, respectively. *Lanes 3–6*, increasing amounts of Dna2. *C*, inhibition of Dna2 nuclease by Dna2 antibody. *Lane 1*, boiled substrate; *lane 2*, preserum but no Dna2 protein, with substrate labeled at the 3′ end. *Lanes 3–7* contain Dna2 plus increasing amounts of preserum, and *lanes 9* and *10* contain Dna2 plus increasing amounts of antibody. *Lane* 8, Dna2 antibody, no Dna2 protein. *D*, circular M13mp18 DNA as nuclease substrate. *Lane 1*, no Dna2; *lane 2*, mock-infected control; *lanes 3–7*, increasing amounts of Dna2.

strain even without induction (glucose plate). We conclude that when Dna2 is cloned on a multicopy plasmid there is sufficient expression for complementation of *dna2–1* even under glucose repression. None of the remaining genes, E640A, P504S, D657A, E675A, Y640A, or K1080E, complemented the *dna2–1* mutant at 37 °C on glucose plates (Fig. 6, *A* and *E*).

When the various *DNA2* genes were induced with galactose (Fig. 6*B*), both the wild type and the P504S protein were able to restore growth to the *dna2–1* mutant strain at 37 °C, whereas the K1080E, E640A, D657A, E675A, and Y693A mutants were unable to complement. (Only K1080E and E640A are shown here, but see Fig. 6*F*.) Complementation by high levels of mutant dna2–1 protein indicates that there is some residual active dna2–1 protein at 37 °C, and simply increasing the amount allows cell growth. Since none of the other proteins complemented under any conditions, these are likely loss of function mutations.

In addition to being loss of function mutations, the E640A, D657A, E675A, and Y693A mutations are dominant negative when the proteins are overproduced (Fig. 6, *C*, *D*, and *F*). Overexpression of the E640A protein at 37 °C in a wild type strain (Fig. 6*C*) and at the permissive temperature in *dna2–1* strains (Fig. 6*D*) is lethal. The relative plating efficiency on galactose *versus* glucose for the *dna2–1* strain expressing the

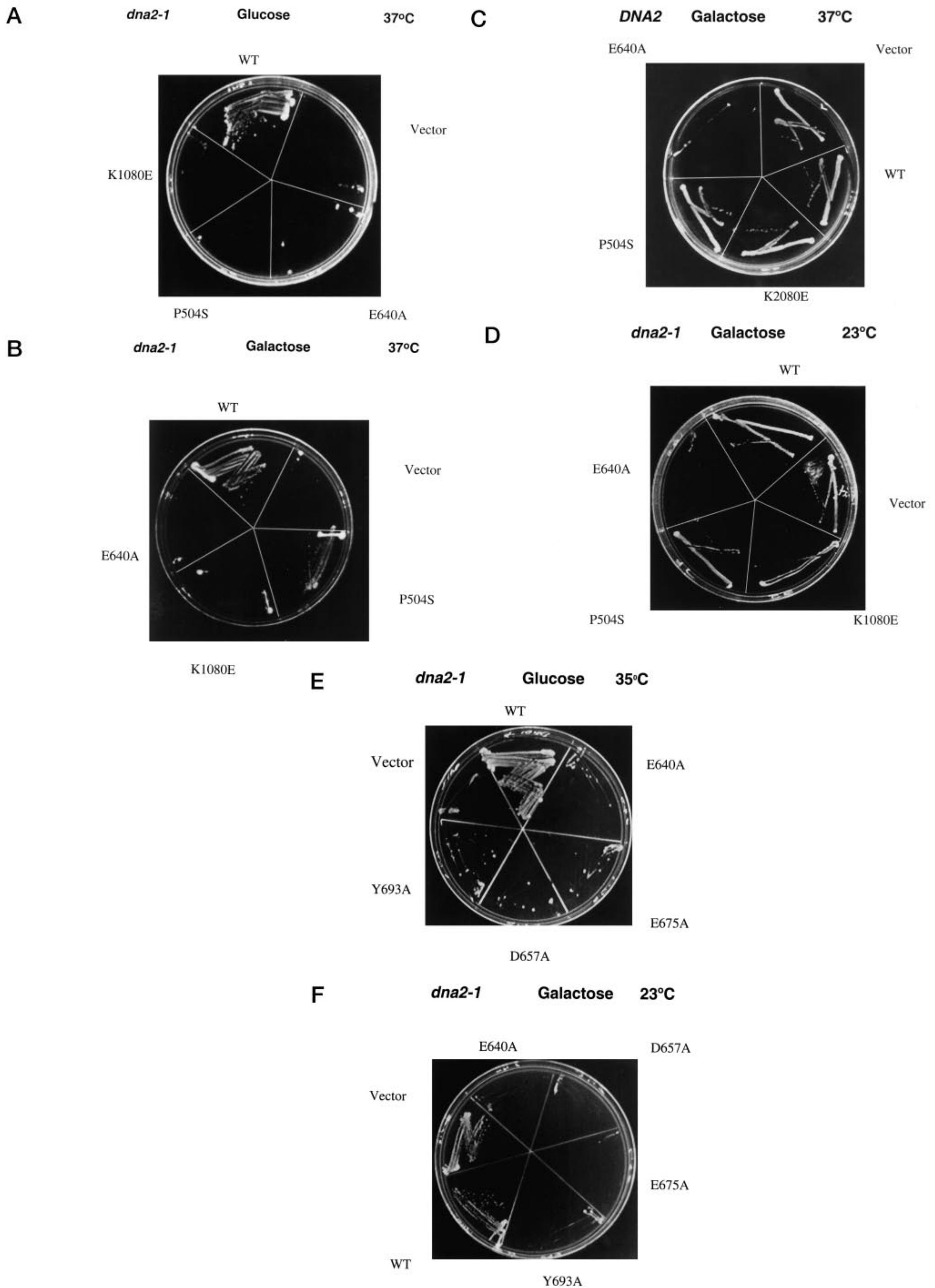

FIG. 6. **Effect of *dna2* mutations *in vivo*.** Growth and x-ray sensitivity of yeast *dna2–1* and *DNA2* strains transformed with plasmids pGAL18, pGAL:Dna2, pGAL:Dna2:E640AA, pGAL:Dna2:P504S, and pGAL:Dna2:K1080E was measured on either glucose- or galactose-containing plates.

TABLE II
Dominant lethality of overexpression of dna2, E640A, in the dna2–1 mutant

Strain 4X154-2D was transformed with the above plasmids, grown in uracil-deficient glucose media to log phase, serially diluted and plated on uracil-deficient, glucose or raffinose/galactose-containing media.

Plasmid	Ratio survival on galactose/glucose at 23 °C
pGAL18	1.02
pGAL18:DNA2	0.97
pGAL18:DNA2:P504S	0.95
pGAL18:DNA2:K1080E	0.94
pGAL18:DNA2:E640A	0.0003

E640A mutant from the *GAL* promoter was quantitated (Table II) and found to be 3×10^{-4}, whereas the relative plating efficiency of plasmid free *dna2–1* cells or *dna2–1* cells carrying plasmids expressing the P504S and K1080E mutants was unity (Table II). The D657A, E675A, and Y693A mutations are also lethal in *dna2–1* plated on galactose at the permissive temperature (Fig. 6*F*). The ability to function as dominant negatives can be explained if the mutant proteins can assemble in replication complexes but are inactive.

Sensitivity of Mutants to X-rays—*dna2–1* and *dna2–2* mutants are sensitive to x-rays and to methylmethane sulfonate (4, 5). Since this x-ray sensitivity is suppressed by overproduction of FEN-1 (5), and since the nuclease activity of Dna2 is similar to that of FEN-1, we tested the ability of the new mutants to restore x-ray resistance to *dna2–2* mutants. The wild type but not K1080E or E640A rescued the x-ray sensitivity of *dna2–2*. Thus, as shown in Fig. 7, the activities affected by mutations in both the C-terminal ATP domain and the N-terminal domain are required for efficient repair of x-ray damage. As shown under "Experimental Procedures," the *dna2–2* strain used here also contained a mutation in an additional helicase, *sgs1Δ*. However, *sgs1Δ* mutants are not sensitive to x-rays (26).

Expression of Mutant Proteins in Yeast and Definition of the Enzymatic Activities of the Mutant Proteins—The *DNA2* genes were expressed in yeast, rather than in insect cells, so that the helicase activity could also be monitored. To allow for two affinity purification steps, the Dna2 proteins were fused to a 6xHis tag at the C terminus and an HA tag at the N terminus as described under "Experimental Procedures." As illustrated in Western blots shown in Fig. 8*D*, the wild type, E640A, P504S, and K1080E proteins were expressed and recovered after purification at approximately equal levels. The other proteins were expressed at similar levels (data not shown).

DNA-dependent ATPase—Ni^{2+}/NTA affinity-purified proteins (see Experimental Procedures) were assayed for DNA-stimulated ATPase at 23 °Cand 37 °C. As shown in Fig. 8*A*, the Dna2 and E640A proteins have nearly equivalent levels of ATPase. The K1080E protein, as expected, is defective (9). The K1080E curve serves as control to demonstrate that Dna2 is the only ATPase in the purified fractions. What was surprising is that the activity of the P504S (the *dna2–1* mutation) protein is only slightly greater than the K1080E protein, since the P504S mutation does not map to the predicted ATPase domain. The P504S protein does not appear to be temperature-sensitive, but rather is defective at all temperatures. The reduced ATPase activity of the N-terminally located mutation suggests that the N terminus may be a positive effector of the ATPase

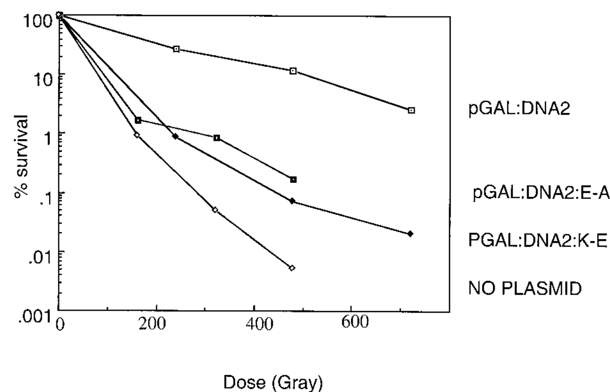

FIG. 7. **Ability of various *dna2* mutant genes to rescue x-ray sensitivity of yeast *dna2–2* strains.** *dna2–2* cells transformed with the plasmids indicated on the *right* were harvested in log phase, irradiated, serially diluted, and plated on uracil-deficient glucose-containing plates, as described (5, 50). The plasmids are high copy number plasmids, which allow expression of the various Dna2 proteins with glucose.

activity of Dna2p, perhaps a DNA binding domain. Although global unfolding cannot be ruled out, it is worth noting that the P504S protein is active *in vivo* when overproduced, suggesting that at least *in vivo* the residual activity is sufficient for function.

To demonstrate that the ATPase of the P504S mutant was partially active, Ni^{2+}/NTA-purified Dna2 and the P504S protein were immunoprecipitated with CL-4B beads coupled with 12CA5 in order to further purify and concentrate the protein. The additional purification step removed the small fraction of non-DNA-dependent ATPases from the Dna2 so that longer incubation times and higher specific activity ATP could be used in the assay. When this preparation was assayed, and the ATP concentration was reduced to 50 μM, the P504S protein now showed approximately 50% the extent and rate of ATPase activity as wild type, whereas the K1080E mutant remained inactive (Fig. 8*C*).

As shown in Fig. 8*B*, the Glu^{675} and Tyr^{693} mutants show similar, though slightly reduced ATPase activity compared with this preparation of wild type. The Asp^{657} mutant shows a lower rate and extent, about 40% of wild type. The significance of these small differences is not clear since the helicase of these mutants does not appear different from wild type (see Figs. 9 and 10). There could be variations in recovery of the proteins that prevent accurate quantitation within a 2-fold range.

DNA Helicase and Nuclease—In order to test the mutant proteins for the helicase and nuclease activities, Dna2 proteins were purified from the Ni^{2+}/NTA column and immunoprecipitated with 12CA5 antibody. Note that these purification steps are different from those used previously and that two affinity purification steps are employed rather than one (2, 9). As shown in Fig. 9 (*lane 5*), the reaction containing Dna2 and ATP yields three bands: a major one of 38 nt, a minor product of approximately 34 nt, and a major product consisting of oligonucleotides of products of approximately 10 nt. We propose that the 38-mer is due to helicase activity acting at the fork between the 5′ tail and the duplex region. Controls show that in the absence of Dna2 protein, no helicase is observed (Fig. 9, *lane 12*). In the presence of Dna2 and absence of ATP, there is also no helicase activity (Fig. 9, *lane 4*, no 38-nt band).

A, top. Strain 4X154–2D, *dna2–1*, transformed with the plasmids carrying the *dna2* alleles indicated, and grown on glucose containing plates at 37 °C. *B*, *bottom*. Strain 4X154–2D, *dna2–1*, transformed with the indicated plasmids, and grown on raffinose/galactose-containing plates at 37 °C. *C*, dominant negative effect of E640A. Strain BJ5459,DNA2, transformed with indicated plasmids and grown at 37 °Con raffinose/galactose-containing plates. *D*, strain 4X154–2D, *dna2–1*, transformed with the indicated plasmids and grown at 23 °C. *E*, strain 4X154–2D, *dna2–1*, transformed with the indicated plasmids and grown at 35 °C. *F*, *dna2–1* transformed with the indicated plasmids and grown at 23 °C.

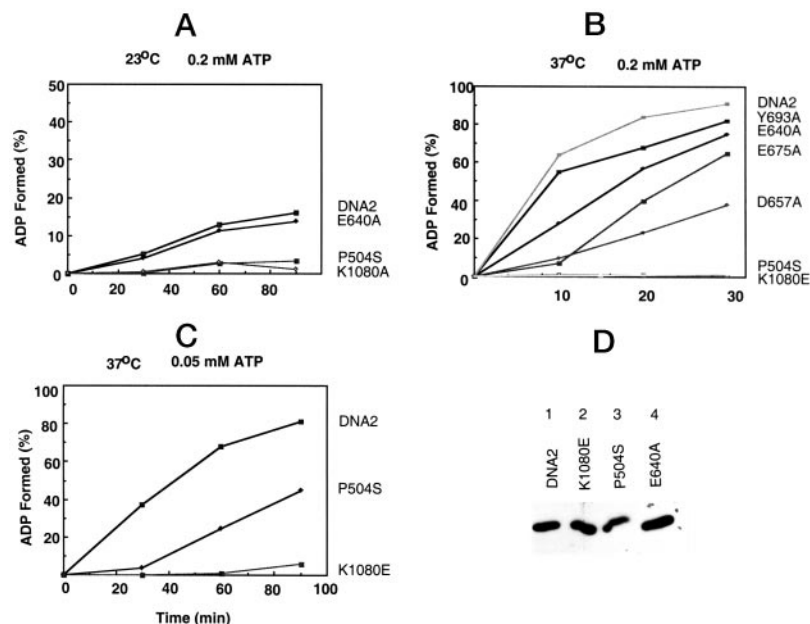

FIG. 8. **DNA-stimulated ATPase activity of wild type and mutant Dna2 proteins purified from yeast.** *A* and *B*, ATPase assays of Ni^{2+}/NTA-agarose-purified proteins. Assays contained ATP at 0.2 mM and 1 μg of Ni^{2+}/NTA-agarose-purified protein. The temperature of incubation is indicated. *C* and *D*, ATPase assays of double immunoaffinity-purified proteins. 5 μg of Ni^{2+}/NTA-agarose-purified protein was immunoprecipitated with 5 μg of 12CA5 coupled to CL-4B beads. Beads were washed and assayed as described under "Experimental Procedures." In *panel C*, the ATP concentration is 0.05 mM and the protein was purified further by immunoprecipitation with 12CA5 antibody as described under "Experimental Procedures." *D*, level of expression of wild type and mutant Dna2 proteins in yeast. His-tagged, HA-tagged Dna2 and the indicated mutant proteins were purified from extracts of yeast through Ni^{2+}/NTA-agarose, loaded onto a 7.5% acrylamide gel, and blotted onto nitrocellulose. Filters were probed with the 12CA5 anti-hemagglutinin antibody.

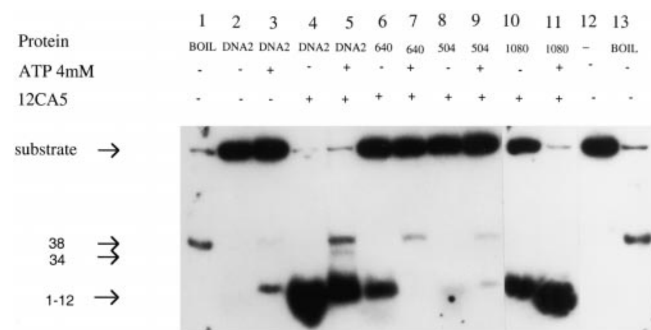

FIG. 9. **Comparison of DNA helicase activities of wild type and mutant Dna2 proteins purified from yeast.** *A*, 5 μg of Ni^{2+}/NTA-agarose-purified protein was immunoprecipitated with 5 μg of 12CA5 anti-HA antibody, followed by addition of 10 μl of 10% protein A beads. Beads were washed and assayed with oligonucleotide hpr3 hybridized to M13 as described under "Experimental Procedures." The contents of each reaction are shown *above* each lane. The size of the bands is indicated on the *right* in nucleotides. *Lane 1*, boiled hpr3; *lane 2*, Dna2, minus antibody; *lane 3*, Dna2, minus antibody, 4 mM ATP; *lane 4*, Dna2, plus 12CA5; *lane 5*, Dna2, 12CA5, 4 mM ATP; *lane 6*, Dna2E640A, 12CA5; *lane 7*, Dna2E640A, 12CA5, 4 mM ATP; *lane 8*, Dna2P504S, 12CA5; *lane 9*, Dna2P504S, 12CA5, 4 mM ATP; *lane 10*, Dna2K1080E, 12CA5; *lane 11*, Dna2, K1080E, 12CA5, 4 mM ATP; *lane 12*, no protein, no antibody; *lane 13*, boiled.

We propose that the 34-mer is due to helicase at the 3′ end of the duplex followed by nucleolytic degradation of the exposed 3′ single-stranded region, since it is not seen in the minus ATP reaction (Fig. 9, *lane 4*). This activity was suppressed in our former work by the introduction of a thiophosphate ester in the 3′ terminal phosphodiester bond of the oligonucleotide (2), but was reported by Bae *et al.* (11).

The much more abundant labeled product migrating with oligonucleotides of 14 nt or less in length indicates that Dna2 nuclease activity cuts the 5′ flap region in the presence of ATP. This nuclease activity is not only ATP-independent (*lane 4*), it is actually inhibited by ATP (compare *lanes 4* and *5*). In the mock immunoprecipitate, that is, in the absence of 12CA5, a small amount of helicase and nuclease activity is observed, but much less than in the presence of antibody. It is likely a small amount of protein precipitates in the presence of high concentrations of BSA that are needed to stabilize the ATPase activity and helicase activity in the purified fraction. BSA is not needed when crude Dna2 is immunoprecipitated, probably because other proteins in the extract serve to stabilize Dna2 (data not shown).

The E640A mutant retains significant helicase activity in the presence of ATP (Fig. 9, *lane 7*, 38-nt band). The helicase is approximately 60% as active as wild type, estimated from three experiments, consistent with the estimated reduction in ATPase of the E640A protein compared with wild type (see Fig. 8). The E640A change has a much greater effect on the nuclease activity than on the helicase and ATPase. The E640A mutant shows less than 15% of the amount of endonuclease as wild type in the absence of ATP (*lane 6*) and has no detectable nuclease activity in the presence of ATP (*lane 7*). This is even more clear evidence than that presented in Fig. 5 with the insect cell preparation of Dna2, that the nuclease is regulated by ATP.

Nuclease Activity of Mutations Affecting the RecB Homology Domain—Mutations in the residues conserved in RecB nuclease have a greater effect on the nuclease than the E640A change. D657A and E675A show no detectable nuclease activity either in the absence or in the presence of ATP (Fig. 10*A*). In these assays we have used a more sensitive denaturing gel nuclease assay than was used in the experiment in Fig. 9 to refine the analysis of the products. Products range from 14 nt down to a few nt. The Y693A mutant protein shows some, although reduced, nuclease activity in the absence of ATP, but no activity in the presence of ATP (Fig. 10*A*), similar to E640A (Fig. 9). Overexposure of the Dna2 protein lane (*panel* on the *right*) shows that wild type nuclease is inhibited by ATP but also shows that there is some ATP-dependent cutting (faint bands between 32 and 14 nucleotides in length). We propose that the ATP binding domain can modulate the activity of the nuclease, since nuclease is always reduced or altered in the presence of ATP, and is completely abolished by ATP in the E640A and Y693A mutants.

Helicase Activity Is Intact in All Four Nuclease Mutants—An important point is that the E640A, D657A, E675A, and Y693A mutant proteins, although deficient in nuclease, are all active helicases (Fig. 10*B*). Thus, the inability of these mutants to support growth (Fig. 6, *A–F*, Table II) is clearly due to a nuclease defect. In addition, the inability of the E640A mutant to complement the x-ray repair defect in a *dna2–2* mutant is also likely due to the nuclease deficiency (Fig. 7). This ability to selectively inactivate nuclease but not helicase also shows that

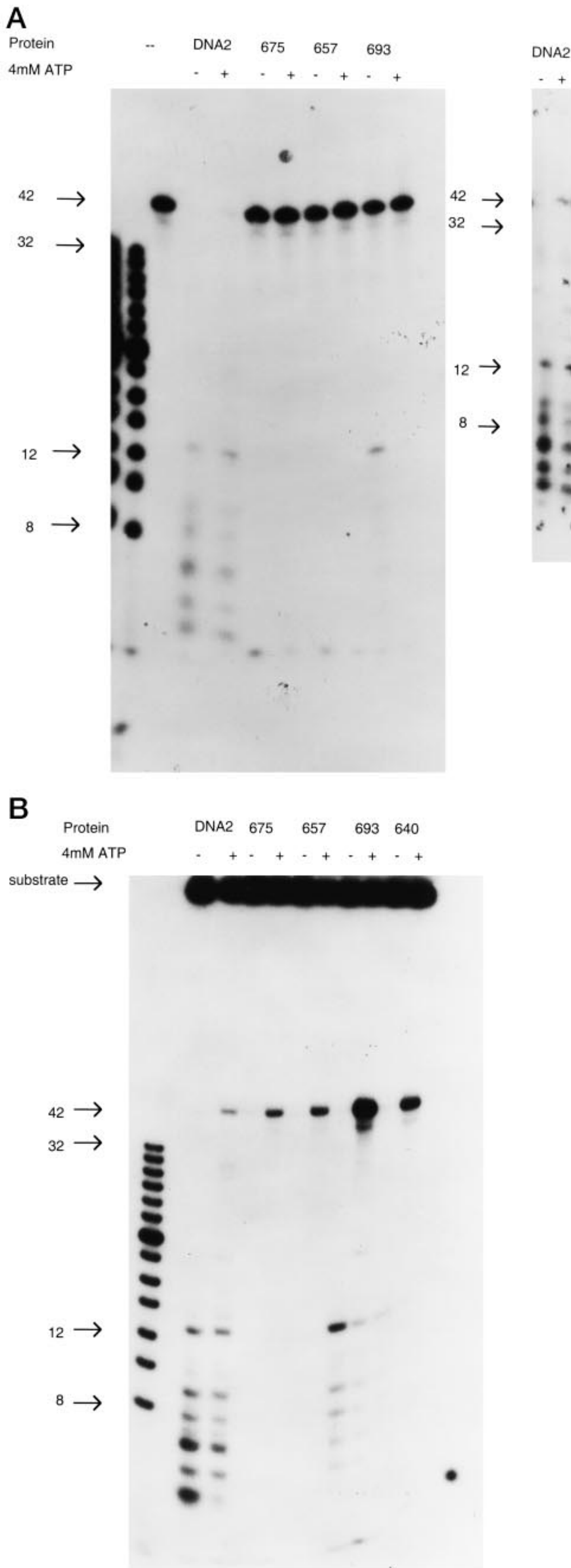

FIG. 10. **Nuclease and helicase assays of N-terminal, RecB-homologous mutants.** *A*, nuclease assays. Nuclease assays were carried out in reaction mixtures as described under "Experimental Procedures" using mutant protein purified by Ni^{2+}/NTA and 12CA5 affinity steps. The substrate was hpr7-labeled at the 5′ end with polynucleotide kinase and hybridized to M13. Products of the reaction were boiled and analyzed on 18% denaturing polyacrylamide gels. Markers are a labeled oligonucleotide ladder from 8 to 32 nt in length, with intervals of two nucleotides between. The *panel* at the *right* is a longer exposure of the Dna2 protein lane, illustrating weak ATP-dependent cutting. The protein in each reaction is indicated at the *top* of the figure. Each protein was assayed in the absence (−) and presence (+) of 4 mM ATP. *B*, helicase activity. Immmunoprecipitates as in *A* were assayed as described under "Experimental Procedures" for helicase activity. The substrate was 42-nt oligonucleotide hpr7 hybridized to M13. Samples were analyzed on an 18% non-denaturing gel.

the helicase activity of Dna2 on forked molecules is not dependent on the nuclease. We are aware in drawing these conclusions that there is a small reduction in DNA-dependent ATPase in some of these mutants (Fig. 8). However, others have provided convincing evidence that full ATPase activity is not required for viability (4).

The availability of the E675A mutant allowed us to reinvestigate the K_m for ATP of the Dna2 helicase activity in the absence of nuclease. Using the assay conditions described in Fig. 10*B*, we determined a K_m of between 70 and 100 μM, similar to that for ATPase. The 4 mM ATP K_m described previously for the wild type enzyme might be required to inhibit the nuclease so that helicase can be observed (2).

Helicase Is Deficient in the P504S and K1080E Mutants—The Dna2 P504S protein, which gives a temperature-sensitive *in vivo* phenotype, retains helicase activity, although like its ATPase activity, the helicase activity is significantly reduced compared with wild type and E640A (Fig. 9). The P504S mutant also retains nuclease activity, although it is reduced by about 95% compared with wild type (Fig. 9, compare *lanes 8* and *4*). The effect of this mutation is different from that of the E640A and Y693 mutations, since the nuclease activity is not completely abolished in the presence of ATP, although it may be slightly further reduced (*lane 9*). Since the P504S mutation is temperature-sensitive, the mutation may destroy the conformation of the protein, rather than identifying amino acids important for one or the other of the activities of Dna2 protein.

For the K1080E protein, helicase is not observed but nuclease is retained at high levels in the presence of ATP, consistent with our previous results (9). The Dna2 K1080E mutant retains full nuclease activity (compared with wild type) in the absence of ATP, but unlike the other Dna2 proteins, the K1080E nuclease activity appears to be stimulated by ATP. We do not know if there is another ATP binding site in this protein, but this stimulation is reproducible.

DISCUSSION

Since Dna2 is a multifunctional enzyme, it has been important to determine which of the enzymatic activities is required for the functions of the protein in the cell. We have previously shown that the ATPase, and presumably the helicase, is essential. We have now shown by generating separation of function mutations that the associated single-strand specific endonuclease activity is integral to the Dna2 protein and that it is also essential for the viability of yeast. Mutations in a conserved region of the N terminus abolished nuclease activity and only helicase activity remained. Changing either of two amino acids in the RecB homology region abolished nuclease in both the presence and absence of ATP, suggesting that the homology has functional significance. The nuclease mutants failed to complement the growth defect of a *dna2–1* mutant and showed a dominant negative affect on growth. This suggests that the four mutant proteins can associate with the essential replication complexes *in vivo*. Thus, we propose that it is the impaired enzymatic activity that is giving rise to repair and replication phenotypes rather than a defect in protein/protein interactions. Although it has previously been proposed that only the helicase

TABLE III
Summary of phenotypes of dna2 mutants

	Complements *dna2–1*	Dominant negative	Repair of x-ray damage	ATPase	Nuclease	Helicase
DNA2	+	−	+	+	+	+
dna2, K1080E	−	−	−	−	+	−
dna2, D657A	−	+	ND[b]	+	−	+
dna2, E675A	−	+	ND	+	−	+
dna2, Y293A	−	+	ND	+	−	+
dna2, E640A	−	+	−	+	−	+
dna2, P504S	+/−[a]	−	TS[b]	+/−	+/−	+/−

[a] Complements the *dna2–1* mutant at 37 °C but only when overproduced.
[b] ND, not determined.
[c] Sensitive to x-rays at 30 °C but not at 23 °C.

is essential for Dna2-mediated repair of damage due to double-strand breaks (4), the nuclease defective E640A mutant is unable to rescue the x-ray repair defect of *dna2–2* mutants.

The nuclease active site of Dna2 appears to map to the N-terminal part of the protein in the RecB homology domain and can be separated from the ATPase domain, in the sense that nuclease-inactivating mutations do not appear to diminish ATPase activity. It has been shown previously that mutations affecting the ATPase domain lie in the C terminus of the protein that is also essential for viability. Nevertheless, the ATPase and helicase activities of the N-terminal mutant, P504S, are reduced, suggesting that the N terminus and the C-terminal ATPase domain may somehow interact. This conclusion is also supported by the inhibitory effect of ATP on the nuclease activity in wild type Dna2 and two of the N-terminal mutants, as well as the ability of ATP to stimulate the nuclease of the P504S mutant. A summary of the phenotypes of the mutants and the activities of the mutant proteins studied here is provided in Table III.

FEN-1 prefers to cleave a 5′ tail adjacent to a duplex region at bases distinct from the single-strand double-strand junction when the region 5′ to the flap structure is single-stranded, although on other substrates it cleaves at the junction (12, 13). Our results show that Dna2 shares this specificity, and this might explain how the two proteins could compensate for each other in DNA replication and double-strand break repair. FEN-1, however, does not cut single-stranded oligonucleotides, whereas Dna2 does cut single-stranded DNA (12, 13, 27). Thus, Dna2 has both structure-specific activities and simple endonuclease activity, whereas FEN-1 is structure-specific. The *in vivo* substrates of the two proteins may overlap, since all single-stranded DNA in the cell is linked to double-stranded DNA.

Although DNA helicase activity is observed with Dna2 expressed in *S. cerevisiae*, helicase is weak or unobservable in baculovirus expressed yeast Dna2 or baculovirus expressed *X. laevis* Dna2 (8, 11). Nevertheless, the observation of helicase at high levels of recombinant protein (11), the absence of DNA helicase in the K1080E protein (expressed in yeast), as well as the association of helicase with six other mutant forms of Dna2 (see Figs. 9 and 10*B*) and under two different purification regimes continue to suggest that a helicase activity is intrinsic to Dna2 and not the result of a co-migrating protein that is removed by the K1080E mutation. Proof of this point will require finding conditions that restore more efficient helicase activity to the Dna2 produced in insect cells. Dna2 may require additional proteins, present only in yeast, for optimal helicase activity. Given the inhibition of *nuclease* activity by ATP and by analogy to RecBC helicase/nuclease, we favor a model in which there may be an inhibitor of the nuclease associated with Dna2 purified from yeast that allows detection of the helicase. For RecBC helicase/nuclease, an inhibitor of the nuclease converts the enzyme from a nuclease into an active helicase. However, there is no evidence for this with Dna2 as yet.

Another possibility is that Dna2 requires a yeast-specific modification for helicase activity. G_1-specific cyclin-dependent kinase (28) and Tor1 DNA-dependent protein kinase (3) both show genetic interactions with Dna2 and therefore are candidates for Dna2 regulators. Yet a third protein kinase, casein kinase 1, Hrr25, which regulates DNA double-strand break repair (29, 30), may also modify Dna2, which we have shown participates in double-strand break repair (5). In *S. pombe*, overexpression of Dna2 and Hhp1, a homolog of Hrr25, suppresses the temperature-sensitive phenotype of *cdc24* (discussed below) (31).

Role of the Dna2 Helicase/Nuclease in Okazaki Fragment Metabolism: Direct or Indirect?—DNA synthesized at the restrictive temperature in *dna2–1* strains is severely fragmented (2). This and the genetic and physical interactions with FEN-1 originally led us to propose that Dna2 functions in Okazaki fragment processing (10). To join nascent fragments, the RNA-containing primer must be removed to prepare for ligation of DNA into continuous chains. The specificity of the Dna2 enzymatic activities can be at least partially reconciled with a role in primer removal. Dna2 can remove a 5′-flap structure, as we have shown here. Dna2 prefers a single-stranded tail adjacent to a duplex region and is less active on a substrate with short stretches of single-stranded DNA flanked by duplex DNA (11). An unwinding event, however, is required to produce a substrate for the flap endonuclease, be it Dna2 or FEN-1 or one of the many other endonucleases found in the yeast genome. Dna2 possesses (or copurifies with) a helicase activity that can unwind a short segment of duplex DNA (24 nucleotides) at a forked junction, but Dna2 helicase cannot unwind fully duplex substrates (2). It is therefore not clear how Dna2 could process the 5′ end of an Okazaki fragment, unless one invokes displacement of the RNA by the oncoming polymerase, as has been proposed by Murante *et al.* (27, 32, 33).

Indirect Role for Dna2 in Lagging Strand Events—Several observations make a direct role in Okazaki fragment processing more difficult to support. The potent single-stranded endonuclease associated with Dna2 would appear to be antagonistic to lagging strand integrity. Second, *rad27*Δ*exo1* mutants and *rad27mre11* mutants are, like *rad27*Δ*dna2* mutants, synthetically lethal. Exo1 is a 5′ to 3′ double-stranded exonuclease that interacts with Msh2 in two hybrid assays (34, 35). Mrell is also a nuclease. Thus, either of these might be a backup for *rad27*. Overexpression of Exo1 did not suppress the temperature-sensitive phenotype of *dna2–1* mutants and *dna2–1exo1* double mutants are not synthetically lethal.[3] If Exo1 processes Okazaki fragments in the absence of FEN-1, then what is the role of Dna2? We must more seriously examine the idea that Dna2 may play a role, which is essential in every cell cycle, in correcting errors made by FEN-1. Failure of FEN-1 to properly process Okazaki fragments may lead to broken replication forks, and the function of Dna2 may involve repairing such collapsed forks. *rad27*Δ *rad52*Δ strains are inviable as a likely consequence of the inability of cells to repair *rad27*-dependent errors by recombination (19). Broken replication forks (and double-strand breaks) are, at least in some cases, repaired by a mechanism that involves the assembly of new replisomes in an origin-independent reaction. In bacteria, an enzyme with activities similar to Dna2, the PriA 3′ to 5′ helicase, is involved in assembling the new primosome (36, 37). This may occur at collapsed replication forks, where the replisome has disassembled or where double-strand breaks occur as forks encounter DNA damage. In yeast, DNA polymerase α, primase, and DNA

[3] M. E. Budd, W.-C. Choe, and J. L. Campbell, unpublished results.

polymerase δ are also involved in recombinational repair of double-strand breaks (38). This may also be related to the break-induced replication described by the latter workers (39). It is interesting to note that *dna2* mutants are sensitive to agents that cause double-strand breaks, such as x-rays and bleomycin, and that this sensitivity is suppressed by overproduction of FEN-1 (5). Overproduction of FEN-1 may simply allow it to function more efficiently, reducing the need for Dna2 on the lagging strand and freeing it for repair of the exogenously induced damage. *dna2* mutants also have a hyperrecombination phenotype, suggesting double-strand breaks are not being efficiently repaired leading to increased recombination (3). Support for a role for Dna2 in repair of broken replication forks also is found in other organisms. *S. pombe cdc24* has been implicated in repair of collapsed replication forks. Arrest of *cdc24-G$_1$* strains at the restrictive temperature is followed by loss in viability and chromosome fragmentation (7). The fragmented chromosomes likely arise from unrepaired broken replication forks. *cdc24* mutants arrest at the restrictive temperature with 2C DNA content (31). The temperature-sensitive growth defect of *cdc24-G$_1$* mutant is suppressed by overproduction of *DNA2* (7), which could be allowing repair of the fragmented chromosomes present in *cdc24-G$_1$* cells at 37 °C. Another allele of *cdc24*, *cdc24-M38*, is suppressed by overproduction of proliferating cell nuclear antigen (PCNA) and replication factor C, establishing a link to the replication apparatus (31).

Other Helicase/Nucleases Involved in DNA Replication and Repair—*E. coli* RecB contains nuclease and helicase encoded in the same polypeptide (40 for review) and is required for recombination-dependent replication in *E. coli* and possibly for repair of DSBs (41). The RecBCD helicase/nuclease is a powerful helicase that unwinds double-stranded DNA at the rate of 1000 bp/s, creating a single-stranded DNA as a substrate for the nuclease. In *E. coli*, broken replication forks can be repaired in a reaction requiring recA, recBCD, priA, dnaB, dnaG, and Ssb and the PolIII holoenzyme (reviewed in Ref. 42). The initial step of repair is catalyzed by RecBCD, which produces a 3′ single-stranded tail for invasion of another duplex. In *E. coli* inhibition of DNA replication results in significant DNA degradation, which is *recB*-dependent (43). Strand invasion is initiated by the helicase activity of RecB when the nuclease is inactivated after encountering a Chi site in the DNA or induction of an inhibitor by the SOS response (44). Dna2 may be involved in such strand invasion. Alternatively, it may be more like priA and be creating the entry site for the replicative helicase and the rest of the replisome (41).

The human Werner syndrome protein is also a helicase with intrinsic nuclease activity (45, 46). The purified protein has a 3′ to 5′ exonuclease activity and 3′ to 5′ DNA helicase activity. A direct involvement in DNA replication is suggested by the observation that S phase is lengthened in Werner cells (47). Werner helicase is homologous to *S. cerevisiae sgs1* and *E. coli recQ*. Sgs1p is a 3′ to 5′ helicase and probably creates a substrate for Top3, perhaps in termination of DNA replication (48). Recently, Sgs1 has been shown to be essential for DNA replication when the *srs2* gene, which also encodes a helicase, is also deleted (49). Both *sgs1* and *dna2* mutants have hyperrecombination and chromosomal instability phenotypes. Unlike Dna2, Sgs1 apparently lacks nuclease activity, and *sgs1-ts srs2*Δ mutants fail to synthesize any DNA at the restrictive temperature, suggesting that Sgs1 and Srs2 helicases are directly involved in moving the replication fork. In summary, three proteins that function as a helicase and nuclease, Dna2, RecBCD, and Werner protein, appear to be required for processing intermediates in replication, which can lead to deletions, amplifications, translocations, and chromosome loss if not resolved.

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