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A yeast gene required for DNA replication encodes a protein with homology to DNA helicases

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ABSTRACT **A yeast gene has been identified by screening for DNA replication mutants using a permeabilized cell replication assay. The mutant is temperature sensitive for growth and shows a cell cycle phenotype typical of DNA replication mutants. RNA synthesis is normal in the mutant but DNA synthesis ceases upon shift to the nonpermissive temperature. The *DNA2* gene was cloned by complementation of the $dna2_{ts}$ gene phenotype. The gene is essential for viability. The gene encodes a 172-kDa protein with characteristic DNA helicase motifs. A hemagglutinin epitope–Dna2 fusion protein was prepared and purified by conventional and immunoaffinity chromatography. The purified protein is a DNA-dependent ATPase and has 3′ to 5′ DNA helicase activity specific for forked substrates. A nuclease activity that endonucleolytically cleaves DNA molecules having a single-stranded 5′ tail adjacent to a duplex region copurifies through all steps with the fusion protein.**

In the absence of eukaryotic *in vitro* replication systems dependent on chromosomal origins of replication, the genetic approach offered by *Saccharomyces cerevisiae* provides an alternative way to identify and analyze the cellular proteins involved in replication. Homologs of the cellular proteins and genes encoding all of the activities required for simian virus 40 leading-strand DNA synthesis have been identified in yeast: DNA polymerase α/primase, DNA polymerase δ and its accessory proteins (RF-C and PCNA), and the three subunits of the single-stranded DNA binding protein RP-A (see ref. 1 for review). A third DNA polymerase, ε, is also required for chromosomal synthesis in yeast, but its specificity for lagging- or leading-strand synthesis is not clear. A yeast origin recognition complex has also been described (2). However, of the major proteins involved in assembling and moving replication forks, a helicase activity has thus far eluded discovery in yeast. It is likely that additional components also remain to be identified. This situation has fostered efforts to identify additional replication genes through isolation of new yeast replication mutants.

We have previously described a screen for DNA replication mutants that monitors DNA synthesis in detergent-permeabilized cells as an assay for DNA replication (3). DNA synthesis in the permeabilized cells is dependent on addition of ATP and all four deoxynucleoside triphosphates. It is thought to represent continuation of synthesis at replication forks active *in vivo* at the time of permeabilization, and indeed the permeabilized cells remain viable and grow when diluted into fresh growth medium. A collection of 400 temperature-sensitive mutants generated by mutagenesis with nitrosoguanidine and previously screened for a specific cell division cycle defect—i.e., arrest with a uniform morphology at the nonpermissive temperature—was used in our study (4). Reasoning that some DNA replication mutants might not arrest uniformly in the cell cycle and thus might have been missed in the *cdc* screen, we grew each strain at the permissive temperature and assayed for DNA synthesis in permeabilized cells at the restrictive temperature. Eighteen strains defective in incorporation of dNTPs into DNA in the *in vitro* reaction were identified. Several of the mutants were alleles of *cdc2*, the structural gene for DNA polymerase δ, verifying that the mutant screen did identify genes required for nuclear DNA replication (3, 5). In this report, we describe the characterization of another mutant defective in DNA replication, originally called *dna154*, that did not fall in any known *cdc* complementation group and that appears to encode a DNA helicase.

MATERIALS AND METHODS

Strains. The following strains were used: A364A, *matahis7 tyr lys2 ade1 gal1 ura1 ade2*; tsdna2, *matadna2 his7 tyr1 lys2 ade1 ura1 ade2*; 3X154-9A, *dna2 gal1 trp1-289 ura3-1,2 can1*; SS111, *matatrp1-289 ura3-1,2 ade 2-101 gal2 can1*; BJ5459, *matura3-52 trp1 lys2-801 leu2Δ1 his3Δ200 pep4Δ his3 prb1Δ1.6R can1*; SEY6210, *mata/α leu1-1, 112/leu1-1,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-901/trpl-901 lys2/lys2 ade2-101/ADE2 suc2Δ/suc2Δ*. SEY 6210 was obtained from Scott Emr (University of California, San Diego). SS111 was obtained from Stuart Scherer (University of Minnesota, Minneapolis). BJ5459 was obtained from the Yeast Genetic Stock Center.

Mapping the Cloned *DNA2* Gene to the *DNA2* Locus. The 1.4-kb *TRP1* fragment from YRp7 was cloned into the *Eco*RI site of YCp154-18-2. The plasmid was cut with *Sna*B1 and transformed into strain SEY6210 by *trp* selection. The diploid was dissected, and a haploid segregant of each mating type was obtained and crossed with strain 3X154-9A *dna2*. Twenty tetrads were dissected from the resulting diploid and 20 parental ditype (PD):O nonparental ditype (NPD):O tetraploid (TT) were observed demonstrating tight linkage of *TRP1* and *DNA2* (PD = 2 *TRP DNA2*:2 *trp dna2*).

Plasmids and Oligonucleotides. The following oligonucleotides were used: HPR1, 5′-GACGTTGTAAACGACGGCCAGTG-3′; HPR2, 5′-GACGTTGTAAAACGACGGCCAGTGATGCTAGTTCTCGA-3′. HPR3, 5′-AGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTG-3′. HPR2-S and HPR3-s are HPR2 and HPR3, respectively, with the 3′-terminal dinucleotide linked by a phosphorothioate bond.

pGAL18 was obtained from Scott Emr and has the *GAL1* and *GAL10* promoter in a *URA3* 2-μm plasmid. YCp154-1 has a 22-kb fragment containing the *DNA2* gene cloned into the *Bam*HI site of YCp50. The gene was cloned from a YCp50 library constructed by Mark Rose (Princeton University, Princeton, NJ). YCp154-2 has a 6-kb *Eco*RI fragment containing the *DNA2* gene cloned into YCp50. YCp154-18-2 has a 4420-bp fragment containing the *DNA2* gene.

pBSDna2 has a 4417-bp fragment containing the *DNA2* gene cloned into the *Eco*RI/*Bam*HI site of Bluescript II SK⁻. pBSDna2HA has the hemagglutinin (HA) epitope MYPYDVPDYASLGGP fused to N-terminal region of the *DNA2*

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Abbreviations: ORF, open reading frame; HA, hemagglutinin.

gene. pGAL18-Dna2HA contains the *DNA2* gene with the N-terminal HA tag cloned into the *Eco*RI site of pGAL18. Site-directed mutagenesis was carried out by the method of Eckstein (6).

RESULTS

Genetic and Physiological Characterization of a DNA Replication Mutant. The temperature-sensitive phenotype of the original *dna154* mutant described in the Introduction segregated 2:2 in crosses and was shown to segregate with the *in vitro* replication defect, suggesting that a single mutation gave rise to both phenotypes (3). The A364a *dna154* mutant was then backcrossed three times with strain SS111. Cell division cycle mutants, by definition, arrest with a uniform morphology, called the terminal phenotype, after incubation at the restrictive temperature. Like other replication mutants (7), after 4 h at 36°C most of the *dna154* cells arrested as dumbbells with an undivided nucleus (Fig. 1). We designate the new mutant *dna2*.

Metabolic labeling studies, carried out as described (8), demonstrated that *dna2* was deficient in DNA synthesis but not in RNA synthesis at the nonpermissive temperature *in vivo* (Table 1). To confirm that the mutation affected DNA replication specifically, replication intermediates formed *in vivo* in the *dna2* strain were analyzed. Both wild-type and *dna2* cells were labeled with [^{3}H]uracil at 23°C and 38°C for 3 h and analyzed by alkaline sucrose sedimentation for size and amounts of newly synthesized DNA as described (9, 10) (Fig. 2). When *dna2* cells were labeled at 23°C, the wild-type and mutant profiles were similar, with a slight reduction in the amount of DNA in the mutant (data not shown). At 38°C, however, no chromosomal sized DNA was synthesized in the *dna2* mutant (Fig. 2). ^{3}H counts were observed only at the top of the gradient, suggesting synthesis of only low molecular weight fragments similar to those produced in *pol1, pol2*, and *pol3* strains at the nonpermissive temperature (5, 9, 10). This is a quick-stop phenotype consistent with a defect in the elongation stage of replication.

Cloning and Disruption of the *DNA2* Gene. The *DNA2* gene was cloned by complementation of the $dna2_{ts}$ mutant. The cloned gene was shown to be genetically linked to *DNA2* by insertion of the *TRP1* gene at the *DNA2* locus and demonstration of linkage of this insertion with the temperature-resistant phenotype.

The *DNA2* gene was disrupted by standard one-step gene replacement in a *ura3/ura3* diploid (11). Disruption was verified by Southern blotting. After sporulation of the *DNA2/dna2::URA3* heterozygous diploid, 2 viable and 2 inviable spores were detected in 14 tetrads dissected. All viable spores were Ura$^-$. Thus, the *DNA2* gene is essential for viability.

Dna2 Protein Contains Helicase Motifs. The DNA sequence of a 4461-bp *dna2* complementing fragment was determined as described in Fig. 3. An open reading frame (ORF) corresponding to a 171,600-Da protein of 1522 amino acids was inferred (Fig. 3). Three different probes, spanning the 4461-bp DNA segment, all hybridized to a 5-kb RNA band (data not shown).

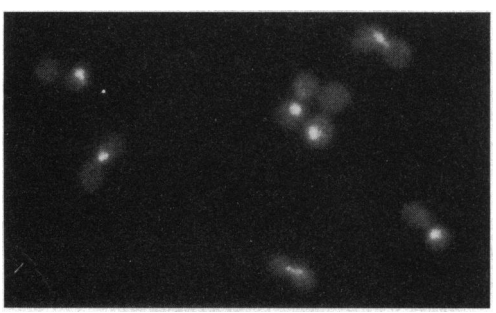

FIG. 1. Terminal phenotype of *dna2* mutant. 3X154 9A cells were incubated at 37°C for 4 h. Cells were fixed with methanol/acetic acid (3:1) followed by staining with 4′,6-diamidino-2-phenylindole.

Table 1. Relative levels of DNA and RNA synthesis at 38°C in wild-type and *dna2* strains

	DNA	RNA
Wild type	1.0	1.0
dna2	0.13	0.9

Values are relative to wild-type values at 38°C. At 23°C, wild type and *dna2* are equal.

The protein contains conserved motifs I, II, III, V, and VI, originally derived from a collection of DNA helicases (Fig. 3) (13, 14). A search of the data base using the N-terminal half of the gene revealed that the entire *DNA2* sequence is more closely related to a human cDNA ORF (accession no. D42046) than to any known yeast sequence. Similarity between the yeast and human ORFs extends over the entire protein, including both the N-terminal domain and the C-terminal helicase region, with 34% overall identity and 45% identity over the C-terminal 400 amino acids. It is therefore possible that the human ORF encodes the human counterpart of *DNA2*.

Isolation of the Dna2 Gene Product. The HA epitope tag MYPYDVPDYASLGGP was fused to the N-terminal region of the Dna2 protein, and the fusion protein was expressed in yeast under control of the *GAL1* promoter. The expression plasmid pGAL18-Dna2HA carrying the HA–*DNA2* gene complemented both the $dna2_{ts}$ and the *dna2*Δ strains. Extracts of cells carrying plasmid pGAL18-Dna2HA revealed a kDa protein on immunoblotting with the 12CA5 HA monoclonal antibody, while cells carrying vector alone showed no cross-reacting material (Fig. 4*A*). The fusion protein was purified to near homogeneity, as described in the legend to Fig. 4, by Western blotting with the 12CA5 antibody to track the protein. A 160-kDa protein identical to that in extracts is seen by using Western blotting of the affinity-purified protein. Only two major species, of 160 and 70 kDa, appear upon Coomassie blue staining (Fig. 4*B*). As a source of control protein for the

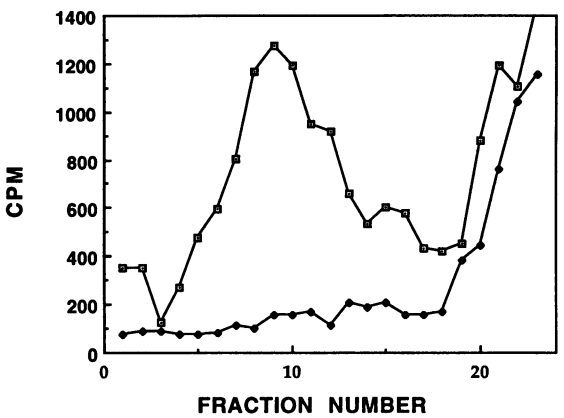

FIG. 2. Alkaline sucrose gradient analysis of residual DNA synthesis in A364A and *dna2* cells. Cells were grown at 23°C to logarithmic phase, labeled with 80 μCi of [^{3}H]uracil (1 Ci = 37 GBq) by growth for 3 h at 38°C, lysed on top of 15–30% sucrose gradients containing 0.3 M NaOH, 0.7 M NaCl, 0.03 M EDTA, and centrifuged in a Beckman SW50.1 rotor at 12,000 rpm for 16 h (9, 10). Fractions (0.2 ml) were collected, acid precipitated, and assayed. RNA was hydrolyzed during the centrifugation. To determine RNA synthesis, 5% of the lysed labeled cells were saved and precipitated with 1 M HCl/0.1 M sodium pyrophosphate. Open symbols, wild type; solid symbols, *tsdna2*. Horizontal axis, normalized fraction with 0 as the bottom and 100 as the top of the gradient. Vertical axis, acid-precipitable ^{3}H cpm. T4 phage sediments at position 16. For clarity, controls labeled at 23°C are not shown.

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MPGTPQKNKRSASISVSPAK KTEEKEIIQNDSKAILSKQT KRKKKYAFAPINNLNGKNTK
VSNASVLKSIAVSQVRNTSR TKDINKAVSKSVKQLPNSQV KPKREMSNLSRHHDFTQDED
GPMEEVIWKYSPLQRDMSDK TTSAAEYSDDYEDVQNPSST PIVPNRLKTVLSFTNIQVPN
ADVNQLIQENGNEQVRPKPA EISTRESLRNIDDILDDIEG DLTIKPTITKFSDLPSSPIK
APNVEKKAEVNAEEVDKMDS TGDSNDGDDSLIDILTQKYV EKRKSESQITIQGNTNQKSG
AQESCGKNDNTKSRGEIEDH ENVDNQAKTGNAFYENEEDS NCQRIKKNEKIEYNSSDEFS
DDSLIELLNETQTQVEPNTI EQDLDKVEKMVSDDLRIATD STLSAYALRAKSGAPRDGVV
RLVIVSLRSVELPKIGTQKI LECIDGKGEQSSVVVRHPWV YLEFEVGDVIHIIEGKNIEN
KRLLSDDKNPKTQLANDNLL VLNPDVLFSATSVGSSVGCL RRSILQMQFQDPRGEPSLVM
TLGNIVHELLQDSIKYKLSH NKISMEIIIQKLDSLLETYS FSIIICNEEIQYVKELVMKE
HAENILYFVNKFVSKSNYGC YTSISGTRRTQPISISNVID IEENIWSPIYGLKGFLDATV
EANVENNKKHIVPLEVKTGK SRSVSYEVQGLIYTLLLNDR YEIPIEFFLLYFTRDKNMTK
FPSVLHSIKHILMSRNRMSM NFKHQLQEVFGQAQSRFELP PLLRDSSCDSCFIKESCMVL
NKLLEDGTPEESGLVEGEFE ILTNHLSQNLANYKEFFTKY NDLITKEESSITCVNKELFL
LDGSTRESRSGRCLSGLVVS EVVEHEKTEGAYIYCFSRRR NDNNSQSMLSSQIAANDFVI
ISDEEGHFCLCQGRVQFINP AKIGISVKRKLLNNRLLDKE KGVTTIQSVVESELEQSSLI
ATQNLVTYRIDKNDIQQSLS LARFNLLSLFLPAVSPGVDI VDERSKLCRKTKRSDGGNEI
LRSLLVDNRAPKFRDANDDP VIPYKLSKDTTLNLNQKEAI DKVMRAEDYALILGMPGTGK
                                                    (vg.pg.gk
TTVIAEIIKILVSEGKRVLL TSYTHSAVDNILIKLRNTNI SIMRLGMKHKVHPDTQKYVP
t)
NYASVKSYNDYLSKINSTSV VATTCLGINDILFTLNEKDF DYVILDEASQISMPVALGPL
                                          (..vde.)
RYGNRFIMVGDHYQLPPLVK NDAARLGGLEESLFKTFCEK HPESVAELTLQYRMCGDIVT
      (gd..q)
LSNFLIYDNKLKCGNNEVFA QSLELPMPEALSRYRNESAN SKQWLEDILEPTRKVVFLNY
DNCPDIIEQSEKDNITNHGE AELTLQCVEGMLLSGVPCED IGVMTLYRAQLRLLKKIFNK
NVYDGLEILTADQFQGRDKK CIIISMVRRNSQLNGGALLK ELRRVNVAMTRAKSKLIIIG
      (t...sqg.e.. .y)                      (valsr..)
SKSTIGSVPEIKSFVNLLEE RNWVYTMCKDALYKYKFPDR SNAIDEARKGCGKRTGAKPI
TSKSKFVSDKPIIKEILQEY ES
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FIG. 3. DNA sequencing reveals helicase motifs in the *DNA2* gene. DNA cloning was carried out according to Sambrook *et al.* (12). BAL-31 deletions were carried out on YCp154-2 by cutting with *Sal* I and treating with BAL-31 followed by addition of *Bam*HI linkers. The C terminus of the gene was cut with *Bam*HI and treated with BAL-31 followed by addition of *Eco*RI linkers. The resulting fragments were cloned into Bluescript and sequenced by the chain-termination method (United States Biochemical). After our determination of the sequence, the same gene was found entered in the NCBI data base as an ORF of unknown function on chromosome VIII (accession no. U00027). The derived amino acid sequence is shown in capital letters. The helicase motifs I, II, III, V, and VI are underlined, and the helicase consensus sequences are indicated in lowercase letters below the corresponding motif. Consensus sequences are from ref. 13 and 14. The helicase motifs of Dna2 protein, but not flanking sequences, are closely related to *SEN1, NAM7,* and *UBF1* (15–17) and to an ORF on chromosome V (accession no. L11229).

following experiments, the extracts of cells transformed with pGAL18 alone were carried through identical purification steps, including the immunoaffinity column.

The Dna2 Protein Has DNA-Dependent ATPase Activity. The hydroxyapatite purified fraction (see legend to Fig. 4) was assayed for ATPase after further purification by immunoprecipitation (Fig. 5). Immunoprecipitates of hydroxyapatite fractions from cells transformed with pGAL18 alone showed no ATPase activity, either in the presence or absence of DNA (lanes 1–3). However, ATPase activity was observed in immunoprecipitates from pGAL18-Dna2HA transformed cells in the presence of DNA (lanes 4 and 5). Little activity was observed in the absence of DNA, showing that the ATPase activity is dependent on or at least stimulated by DNA. Appropriate markers showed that the ATP was hydrolyzed to ADP and, by inference, P_i. These are properties consistent with ATPase activities associated with DNA helicases.

The soluble immunoaffinity-purified fractions, from cells containing pGAL18-Dna2HA or pGAL18 alone, were then assayed directly for ATPase activity. As expected, there was no ATPase in the cells without fusion protein (data not shown). As shown in Table 2, however, the fusion protein contained DNA-stimulated ATPase. ATP and dATP are hydrolyzed more efficiently than the other nucleotides. DNA stimulated the ATPase and dATPase reactions 15-fold. We conclude that ATPase activity is associated with the Dna2 protein.

The Dna2 Protein Has Associated DNA Helicase Activity. The 12CA5 affinity-purified HA–Dna2 fusion protein was next assayed for DNA helicase activity. A set of substrates was prepared by hybridizing three different oligonucleotides to

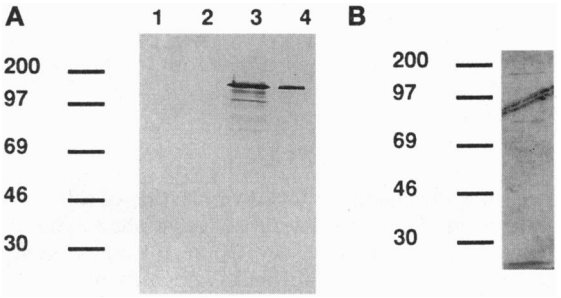

FIG. 4. Purification and gel electrophoretic analysis of HA-tagged Dna2 protein. HA–Dna2 protein was purified from BJ5459 cells transformed with pGAL18-Dna2HA. Transformed cells were grown in 2% synthetic raffinose medium to 10^7 cells per ml, galactose was added to 2%, and cells were harvested after 6 h. Frozen cells (3–4 g) were lysed in buffer containing 10% (vol/vol) glycerol, 0.1 M NaCl, 0.025 M Tris·HCl (pH 7.6), 2 mM dithiothreitol (DTT), by grinding with a mortar and pestle in liquid nitrogen. The powder was thawed, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 μg of pepstatin A per ml, 1 μg of leupeptin per ml) were added. The lysate was centrifuged at 100,000 $\times$ *g* for 20 min. NaCl was added to 1 M and PEG 8000 was added to 6% to remove DNA. After 15 min, extracts were centrifuged at 30,000 rpm for 20 min and the supernatant (180 mg of protein) was loaded onto a 20-ml hydroxyapatite column. The column was washed with 80 ml of 25 mM KH_2PO_4 (pH 7.2), 10% glycerol, and 3 mM DTT, and then eluted with 0.2 M KH_2PO_4 (pH 7.2), 10% glycerol, 3 mM DTT. The 0.2 M wash (30 mg) was passed through a Sepharose CL-4B column and then loaded onto a 1-ml column consisting of CL-4B coupled with the monoclonal antibody 12CA5. The column was washed with 200 ml of TBS + 10% glycerol and then 10 ml of TBS + 10% glycerol and 0.1% Tween. 12CA5 peptide was added (5 mg) in TBS + 10% glycerol and 0.1% Tween. The peptide and column were incubated for 12 h at 4°C. Since only ≈25% of the material loaded was recovered after the first elution with peptide at 4°C, 12CA5 peptide (4 mg) was added again to the column for a second elution following the method of Field *et al.* (18). The column was then incubated at 30°C for 15 min and eluted at room temperature. Western blots showed that both elutions contained approximately equal amounts of the HA–Dna2 protein. Total recovery was 150 μg of protein and represented ≈70% of the crossreacting material loaded. (*A*) Western blot of extracts and first elution from 12CA5 affinity column. Lanes: 1 and 2, 10 and 20 μg of extract of cells containing the pGAL18 vector alone; 3, 10 μg of extract of cells containing the pGAL18-Dna2HA plasmid; 4, elution 1 of the 12CA5 affinity column, ≈500 ng. (*B*) Coomassie-stained gel of the material from the second elution. The second elution is shown because the protein recovered was less active in ATPase and helicase assays, probably due to the 30°C incubation, since more protein could be loaded on the gel, to allow detection of possible contaminating protein. The second elution is likely to be less pure as more total protein is released. However, the first elution, which is used in Fig. 6 and Table 2, looked identical, with ≈50% as much protein loaded (data not shown). Numbers on left are kDa.

M13. HPR1 is a 24-mer complementary over its entire length to M13. HPR2 is a 38-mer, which yields a forked structure with a 3′ noncomplementary tail of 14 nt when hybridized to M13. HPR3 is a 38-mer, which also yields a forked structure after hybridization with M13, but which has a 5′ noncomplementary tail of 14 nt. In the experiments shown, the terminal phosphodiester linkages were through a thiophosphate in hopes of selectively inhibiting exonuclease, but identical results were obtained with standard oligonucleotides. The oligonucleotides were labeled at their 5′ ends using polynucleotide kinase. As shown in Fig. 6, only HPR3 showed a displaced band when incubated with HA–Dna2 protein. In fact, two bands, the expected 38-mer and a band of 14–16 nt, were observed. This suggested that both a helicase and a nuclease specific for the HPR3 substrate but not HPR1 or HPR2 were associated with the tagged Dna2 protein. Neither helicase nor nuclease activity was found in the immunoaffinity-purified fraction from cells carrying the pGAL18 vector alone. The helicase activity can be

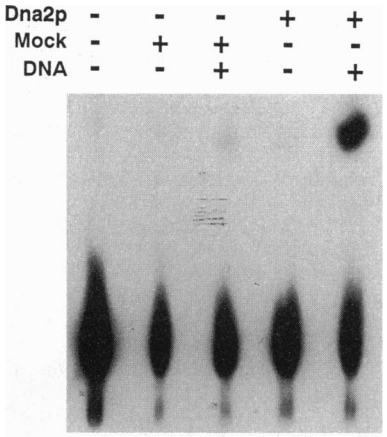

FIG. 5. ATPase activity associated with the HA–Dna2 protein. Immunoprecipitates were prepared by using the hydroxyapatite fraction prepared either from cells carrying the pGAL-Dna2HA plasmid or carrying vector alone, as described in the legend to Fig. 4. Protein fraction (0.2 mg) was mixed with 20 μg of 12CA5 monoclonal antibody for 1 h at 0°C. Twenty microliters of 10% protein A beads was added followed by a 1-h incubation at 0°C. Beads were washed six times with TBS + 0.1% Tween, two times with 2× assay buffer, resuspended in 20 μl of 2× assay buffer, and used for ATPase. Reaction mixtures (40 μl) contained 40 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 0.2 mM ATP, and 2.5 mM dithiothreitol and were incubated at 37°C for 1 h. The reaction was stopped by addition of 8 μl of 20 mM EDTA containing 40 mM ATP and 40 mM ADP, as markers. Reaction mixture (1–2 μl) was spotted on a polyethyleneimine plate and developed with 1 M HCOOH/0.4 M LiCl. The plates were autoradiographed and spots corresponding to ATP and ADP were eluted and assayed. Lanes: 1, no protein; 2 and 3, protein from cells transformed with vector alone in the absence and presence of poly(dA)/oligo(dT) (20:1), respectively; 4 and 5, protein from cells expressing the HA–*DNA2* gene in the absence and presence of poly(dA)/oligo(dT) (20:1), respectively.

distinguished from the nuclease activity, however, since the helicase, unlike the nuclease, requires ATP (or dATP; data not shown) as a cofactor (lanes 6–9). The NTP substrate specificity of the helicase is the same as that of the DNA-dependent nucleoside triphosphatase activity shown in Fig. 5. The apparent K_m for ATP in the helicase reaction was estimated from the experiment shown in Fig. 6 (lanes 10–17) to be ≈4 mM. In the presence of the helicase, the product of the nuclease seems to be slightly longer.

We suggest that the Dna2 protein encodes the ATPase and helicase since the Dna2 protein has helicase motifs. We suggest that the nuclease is a second protein that is tightly associated with the Dna2 protein (see *Discussion*).

DISCUSSION

We have characterized a temperature-sensitive mutant from yeast. The mutant is defective in DNA replication since: (*i*) *dna2* mutants arrest at a specific point in the cell cycle characteristic of all known DNA replication mutants; (*ii*) and (*iii*) *dna2* mutants fail to carry out the elongation stage of

Table 2. NTPase activity of affinity-purified HA–Dna2 protein

	% hydrolyzed	
	+ DNA	− DNA
ATP	82	5.6
dATP	77	5.0
dCTP	8.1	3.0
dTTP	7.3	2.4
dCTP	4.2	4.7

Assays were carried out as described in the legend to Fig. 5.

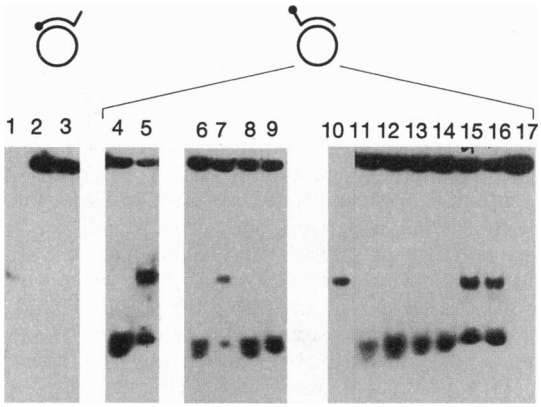

FIG. 6. Helicase activity of affinity-purified Dna2 protein. The helicase assay mixture contained 40 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 2.5 mM dithiothreitol, 4 mM ATP, oligonucleotides labeled with ^{32}P at their 5′ ends hybridized to M13, and 0.1 ng of HA–Dna2 protein (purified through the 12CA5 column step as described in the legend to Fig. 4, first elution). After 15 min at 37°C, the reaction was stopped with 1/5th vol of 40% glycerol, 50 mM EDTA, 2% SDS, 3% xylene cyanol, 3% bromophenol blue and loaded onto 12% polyacrylamide gels, electrophoresed for 1 h at 100 V, and autoradiographed. The sizes of the bands were determined on a longer gel with markers (data not shown) and are given in the text. Lanes: 1, HPR2-S boiled, 1/4 as much as loaded in lanes 2 and 3; 2 and 3, HPR2-S incubated with HA–Dna2 protein in the absence and presence of ATP; 4 and 5, HPR3-S incubated with HA–Dna2 protein in the absence and presence of 4 mM ATP; 6–9, HPR3-S incubated with HA–Dna2 protein and 4 mM GTP, ATP, TTP, and CTP, respectively; 10, HPR3-S boiled (only 1/4 of the reaction mixture was loaded to enhance accuracy of sizing of the oligonucleotide); 11–16, HPR3-S plus 0.5, 1, 2, 4, and 10 mM ATP, respectively; 17, HPR3-S incubated in the absence of HA–Dna2 protein.

replication *in vitro* in cells permeabilized to triphosphates and have a quick-stop DNA synthesis phenotype *in vivo*, as determined by direct precursor incorporation studies; (*iv*) the *DNA2* gene is essential for viability; (*v*) the *DNA2* gene appears to encode a DNA helicase, with a specificity consistent with a role in DNA replication and/or repair.

The sequencing of the essential *DNA2* gene revealed that it does not correspond to any previously identified eukaryotic replication protein or mutant. Instead, the *DNA2* gene encodes a protein that contains helicase motifs in its C-terminal half and an N-terminal half with no significant similarity to any previously identified protein. The importance of the gene is underscored, however, by the recent cloning of a human gene that shares significant sequence similarity with *DNA2* over both the N- and C-terminal domains. The presence of the helicase motifs in *DNA2* led us to carry out biochemical experiments to demonstrate actual ATPase or helicase activity. Purification of an epitope-tagged Dna2 protein from yeast revealed both ATPase and helicase activity associated with the tagged protein. No helicase described to date in yeast has the properties of this helicase (molecular mass, cofactor requirement, K_m for ATP, substrate specificity, direction of movement) (19–23). Judging from the phenotype of the *dna2* mutant, the sequence of the gene, and the activities of the highly purified form of the protein we studied, we propose that the *Dna2* gene encodes this helicase. This conclusion is supported by demonstration of loss of ATPase activity in a *dna2* mutant protein with a mutation in the conserved ATP binding site (unpublished data).

The specificity of the helicase is consistent with a role in DNA replication. Fully hybridized oligonucleotides, even ones as short as 24 bp, are not displaced by the Dna2 helicase. Only molecules that simulate a replication fork are substrates. The fact that HPR3, but not HPR1 or HPR2, is the preferred substrate is consistent with a model in which the helicase binds

to a single strand, which must be longer than 14 nt, and translocates 3′ to 5′ on the strand to which it binds. When a fork is encountered, it is unwound. When a fully hybrid terminus is encountered, however, there is no unwinding. The simplest interpretation of the polarity of helicase translocation is that Dna2 moves in the direction of replication on the leading strand at the replication fork, as has been proposed for T antigen and herpes simplex virus UL9 (24–28). However, another model might derive from the *Escherichia coli* PriA protein, a potent 3′ to 5′ DNA helicase that binds to a specific sequence in single-stranded DNA called the primosome assembly site. Lee and Marians (29) have proposed that PriA may serve as a DNA translocase that helps move the primosome in the 5′ to 3′ direction by pumping the DNA through the protein. PriA, however, binds only to specific sequences, it does not require a forked structure, and it is not essential for chromosomal replication.

Copurification of a nuclease with the tagged Dna2 protein prevents definitive proof that the Dna2 protein functions as a helicase, since the strand displacement activity we observe could be related to the nuclease. Arguing against this is the fact that the helicase but not the nuclease requires ATP hydrolysis. The nuclease is of some interest in itself, however. The nuclease requires a short 5′ single-stranded tail adjacent to a duplex region (unpublished data) and cleaves endonucleolytically adjacent to the junction between the single-stranded tail and the duplex region, giving rise to the 14- to 16-nt product shown in Fig. 6. This substrate specificity is very similar to that of the enzyme encoded by the yeast YKL510 gene (30). YKL510 is 60% identical to the mouse and human FEN1 nucleases (30), the latter being required for lagging-strand synthesis in the simian virus 40 *in vitro* replication system (31). A high copy number plasmid carrying the YKL510 gene suppresses the temperature-sensitive defect of the *dna2* mutant at 36°C (unpublished data). Such suppression suggests that copurification reflects an important physiological interaction. Further work is needed to verify this point, but the association may be a clue to the precise role of the Dna2 protein in replication.

The proposed role for Dna2 as a replicative helicase does not exclude a role for additional helicases in yeast chromosomal DNA replication, including at least one that, like the prokaryotic primosomal helicases, is on the lagging strand. A number of genes with homology to helicases have been studied in yeast. The most closely related is an ORF on chromosome V, as described above. It will be interesting to further define the roles of all of these proteins.

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