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DNA2 Encodes a DNA Helicase Essential for Replication of Eukaryotic Chromosomes*

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Although a number of eukaryotic DNA helicases have been identified biochemically and still more have been inferred from the amino acid sequences of the products of cloned genes, none of the cellular helicases or putative helicases has to date been implicated in eukaryotic chromosomal DNA replication. By the same token, numerous eukaryotic replication proteins have been identified, but none of these is a helicase. We have recently identified and characterized a temperature-sensitive yeast mutant, *dna2ts*, defective in DNA replication, and have cloned the corresponding gene (Kuo, C.-L., Huang, C.-H., and Campbell, J. L. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 30, 6465–6469; Budd, M. E., and Campbell, J. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7642–7646). The *DNA2* gene is essential and encodes a 172-kDa protein with DNA helicase motifs in its C-terminal half and an N-terminal half with no similarity to any previously described protein (Budd, M. E., and Campbell, J. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7642–7646). Here we show that the helicase domain is required *in vivo* and that a 3′ to 5′ DNA helicase activity specific for forked substrates is intrinsic to the Dna2p. The N terminus is also essential for DNA replication. Thus, the structure of this new helicase is different from all previously characterized replicative helicases, which is consistent with the complex organization of eukaryotic replication forks, where the activities of not one but three essential DNA polymerases must be coordinated.

A DNA helicase is a central component of the architecture of prokaryotic DNA replication forks. Reconstitution of the basal apparatus for replication of the SV40 virus has established the requirement for a DNA helicase in eukaryotic DNA replication as well. However, SV40 DNA replication requires only the helicase associated with the viral large T antigen and no cellular helicase. Therefore, we have looked for a cellular replicative helicase using yeast genetic analysis.

Recently, we characterized a gene, *DNA2*, which complements a temperature-sensitive yeast strain defective in the elongation stage of DNA replication (1, 2). The *DNA2* gene is essential for viability and encodes a 1522-amino acid protein, the most prominent feature of which is the presence of the six conserved motifs characteristic of DNA helicases. These motifs are localized to the COOH third of the protein (amino acids 1035–1522). In order to demonstrate that the protein had helicase activity, the HA-Dna2 protein was purified 50,000-fold. The immunoaffinity-purified protein was shown to be associated with a DNA-dependent ATPase and a DNA helicase. Interestingly, the helicase is active only on a substrate with a forked structure, as is true of many prokaryotic and viral replicative helicases and appears to translocate in the 3′ to 5′ direction, the polarity of the leading strand at a replication fork (2).

While these experiments suggest that Dna2p is a replicative helicase, they are preliminary in two ways. First, although mock purifications yield no ATPase or helicase activity, our biochemical approach could not rule out that the ATPase and helicase activities were copurifying with, rather than intrinsic to, the Dna2p. Second, because more than two-thirds of the protein sequence was not conserved in any known helicase and might therefore encode some novel replicative function, our previous results did not allow us to conclude that the essential role of Dna2p in replication was that of a helicase. An example of a DNA-dependent ATPase and helicase whose essential function may not require the helicase activity of the protein is the Rad3 protein. Rad3 is essential for viability and is required for both nucleotide excision repair and for mRNA transcription. When the conserved lysine of the ATP-binding site, GKT, is changed to arginine, the protein loses its DNA dependent-ATPase and helicase. The resulting *rad3* mutant is sensitive to UV irradiation but is viable (3, 4). Thus, the helicase appears to be required for nucleotide excission repair but seems to be dispensable for the essential function of *RAD3* in transcription. In this paper, we show that a helicase activity is intrinsic to the Dna2p and that the helicase is required for its *in vivo* function.

MATERIALS AND METHODS

Strains and Plasmids—Strains used are: 3X154–9A, *dna2 trp1–289 ura3–1,2;* BJ5459, **a** *ura3–52 trp1 lys2–801 leu2Δ1 his 3Δ200 pep4Δ:1–1153 prb1Δ1.6r;* JD52, **a** *leu2–3,112 his3Δ200 trp-1Δ63 ura3–52 lys1–801;* JD53, *α leu2–3,112 his3Δ200 trp1Δ63 ura3–52 lys2–801;* and MB1, **a** *dna2Δ::URA3 ura3–52 leu2–3,112 his3Δ200 trp1Δ63 lys2–801/α DNA2 ura3–52 leu2–3,113 his3Δ200 trp1Δ63 lys2–801.* In the plasmids used pJDgal:DNA2K has the DNA2 gene (amino acids 105-1522) cloned into the *Eco*RI site of pJDgal (5) and pJDgal:DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJDgal at the *Eco*RI site. DNA sequencing verified that only one change had occurred. pGAL18-Dna2HA has the Dna2 gene (amino acids 105-1522) cloned into the *Eco*RI site of pGAL18. All plasmids encode *DNA2* genes with the hemagglutinin epitope fused in frame to methionine 105. The first 105 amino acids are dispensable for function (see text).

Purification of Dna2 Protein—HA-Dna2 protein was purified from BJ5459 cells transformed with pGAL18-Dna2HA or pJDgal:DNA2E. Transformed cells were grown in 2% synthetic raffinose media to 10^7 cells/ml, galactose was added to 2%, and cells were harvested after 6 h. Frozen cells (3–4 g) were lysed in buffer containing 10% glycerol, 0.1 M NaCl, 0.025 M Tris-HCl, pH 7.6, 2 mM DTT,[1] by grinding with a mortar and pestle in liquid nitrogen. A sample (3 g) was thawed, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 2 μg/ml pepstatin A, 1 μg/ml leupeptin) were added. The lysate was centrifuged at 100,000 × *g* for 20 min. NaCl was added to 1 M, and polyethylene glycol 8000 was added to 6% to remove DNA. After 15 min,

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[1] The abbreviations used are: DTT, dithiothreitol; kb, kilobase pair(s); PCR, polymerase chain reaction.

FIG. 1. **Lack of complementation of *dna2-1* by the Dna2E mutant protein.** 3X154 9A *dna2-1 ura3* was transformed with the plasmids expressing the wild-type (*DNA2K*) and ATPase mutant (*DNA2E*) genes, pJDgal:DNA2K or pJDgal:DNA2E. Ura^+ transformants were selected at 23 °C. Both plasmids yielded viable transformants at 23 °C. Colonies carrying the indicated plasmids were then restreaked and incubated at 37 °C in the presence of galactose.

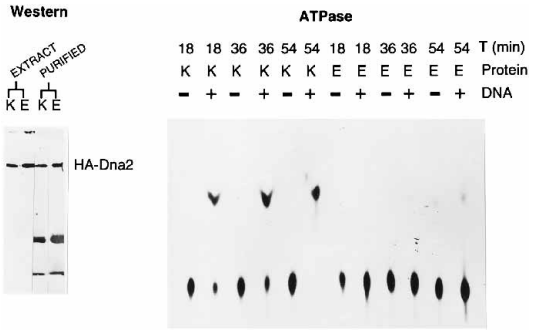

FIG. 2. **Mutation of the conserved ATP-binding site eliminates the DNA-dependent ATPase activity of Dna2p.** Protein (0.2 mg of the 0.2 M hydroxyapatite wash prepared as described under "Materials and Methods") was mixed with 20 μg of 12CA5 monoclonal antibody for 1 h at 0 °C. Twenty microliters of 10% protein A beads was added followed by a 1-h incubation at 0 °C. Beads were washed eight times with TBS/0.1% Tween, 2 times with 2 × assay buffer, resuspended in 20 μl of 2 × assay buffer, and used for Western blot analysis after boiling beads in SDS or directly for ATPase assays. *Left*, the Western blot shows the wild-type (labeled *K*) and the mutant (labeled *E*) proteins in extracts and in the immunoprecipitates used for ATPase assays, as indicated. *Right*, ATPase assays of wild-type and mutant protein were carried out as described under "Materials and Methods" in the presence and absence of DNA, as indicated. *First 6 lanes*, wild-type protein; *last 6 lanes*, mutant protein. About 55 and 100% of the ATP was converted to ADP after 18 and 54 min, respectively, in the presence of DNA by the wild-type protein. No ATPase is observed when extracts of cells carrying the pGAL18 vector alone are carried through the same purification procedure. The spot at the origin (lower spot) corresponds to ATP, and the spot that moves corresponds to ADP.

extracts were centrifuged at 30,000 rpm for 20 min, and the supernatant (25 mg of protein) was loaded onto a 20-ml hydroxyapatite column. The column was washed with 10 ml of 25 mM KPO_4, pH 7.2, 10% glycerol, and 3 mM DTT and then eluted with 0.2 M KPO_4, pH 7.2, 10% glycerol, 3 mM DTT. Protein was then purified by immunoaffinity chromatography as described previously (2).

ATPase Assays—The ATPase reactions (20 μl) contained 40 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 0.2 mM [α-^{32}P]ATP, 2.5 mM DTT, and 0.2 mg/ml poly(dA) as indicated and were incubated at 37 °C for the times indicated. The reaction was stopped by the addition of 8 μl of 20 mM EDTA containing 40 mM ATP, 40 mM ADP as markers. Reaction mix (1–2 μl) was spotted on a polyethyleneimine plate and developed by ascending chromatography with 1 M HCOOH, 0.4 M LiCl. The plates were autoradiographed, and spots corresponding to ATP and ADP were excised and radioactivity determined.

Helicase Assays—The helicase assay measures displacement of a labeled 38-base oligonucleotide hybridized to M13 DNA over 24 bases and having a 14-nucleotide 5′ noncomplementary tail as described (2).

RESULTS AND DISCUSSION

The occurrence of a helicase domain does not in itself mean that it is part of the essential function of a gene. For instance, the helicase activity of the Rad3 protein is dispensable for its role in mRNA transcription (3, 4). To investigate whether the helicase domain of Dna2p contributed to its essential physiological function, the invariant lysine 1080 in the major $GX_4GK(T/S)$ nucleotide-binding loop was changed to glutamic acid. The lysine is essential for binding the β,γ-phosphate of ATP or GTP (6). The ability of wild-type and ATPase mutant genes to complement the *dna2-1*$_{ts}$ strain and a *dna2*Δ deletion strain was then assessed. As shown in Fig. 1, dna2-1$_{ts}$ cells transformed with the Dna2K (wild-type) plasmid are complemented for growth at 37 °C, but cells transformed with the Dna2E (mutant) plasmid are not. Wild-type cells transformed with Dna2E grow normally (data not shown). In order to establish that the mutant Dna2E protein could not support growth when it was the only form of Dna2p in the cell, the *DNA2*/*dna2*Δ strain, used previously to demonstrate that DNA2 was essential (2), was transformed with both wild-type pJDgal:DNA2K and mutant pJDgal:DNA2E plasmids. The transformants were sporulated and tetrads analyzed. Transformants carrying pJDgal:DNA2K gave four viable spores in all tetrads, while pJDgal:DNA2E failed to complement *dna2*Δ spores, giving rise to 2 viable and 2 inviable spores in each tetrad. Interestingly, the *dna2*Δ strain transformed with pJDgal:DNA2K grew on glucose as well as on the inducing carbon source galactose. In contrast, the pJDgal:DNA2K plasmid complemented dna2-1$_{ts}$ strains only after induction on galactose (Fig. 1). This suggests that when expression of the wild-type Dna2 protein is low, the *dna2-1*$_{ts}$ protein may exhibit a dominant negative effect, perhaps by forming inactive heteroallelic oligomers containing a mixture of wild-type and mutant protein. Since the *dna2-1*$_{ts}$ mutation is recessive in a heterozygous diploid and since pJDgal:DNA2K complements the *dna2-1*$_{ts}$ strain when grown with galactose, raising the level of wild-type Dna2 protein eliminates the possible dominant negative effect of *dna2-1*$_{ts}$ protein.

The Dna2E mutant was then used to verify that the Dna2 protein had helicase activity. Both the wild-type (HA-Dna2K) and the mutant proteins (HA-Dna2E) were expressed in yeast as hemagglutinin epitope fusion proteins under the control of the inducible *GAL10* promoter. Dna2p was partially purified, immunoprecipitated with the hemagglutinin monoclonal antibody 12CA5 from the 0.2 M hydroxyapatite eluant, and assayed for DNA-dependent ATPase activity (Fig. 2). The HA-Dna2K protein converted ATP to ADP and P_i, and the activity was dependent on the addition of DNA, as expected for a DNA helicase. The mutant HA-Dna2E protein, however, exhibited no ATPase activity. Similar amounts of the respective HA-tagged Dna2 proteins were shown to be present in both immunoprecipitates, verifying that the mutant protein was expressed at levels equivalent to wild-type protein (Fig. 2). Thus, the Dna2p is a DNA-dependent ATPase.

Both wild-type and mutant protein were purified further and assayed for DNA helicase activity (Fig. 3). We had previously shown that a 3′ to 5′ DNA helicase specific for the forked substrate shown in Fig. 3 copurified from yeast with HA-Dna2p through all steps. Despite many attempts, however, we were

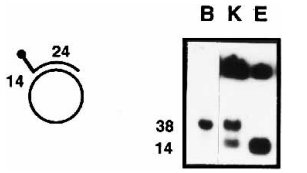

FIG. 3. **Mutation of the conserved ATP-binding site eliminates the DNA helicase activity of Dna2p.** The wild-type and mutant proteins eluted from hydroxyapatite were purified further by immunoaffinity chromatography (see "Materials and Methods" and Ref. 2). Purified protein (10 ng) was assayed for DNA helicase activity. *Lane B*, boiled substrate; *lane K*, wild-type protein; *lane E*, ATP site mutant. The 38 mer is the product of the helicase unwinding reaction. The most rapidly moving bands in *lanes 2* and *3* are 14–16 mers and are due to a structure-specific nuclease activity that copurifies with the helicase. As shown previously, the nuclease does not require ATP hydrolysis and is, therefore, not affected by the mutation. Thus it is either encoded by another gene or resides in a separate domain of *DNA2* (see text). The forked molecule shown on the *right* is the only substrate configuration on which the helicase found in Dna2 preparations is active (2). Neither helicase nor nuclease is observed when extracts of cells carrying the pGAL18 vector alone are carried through the same purification procedure.

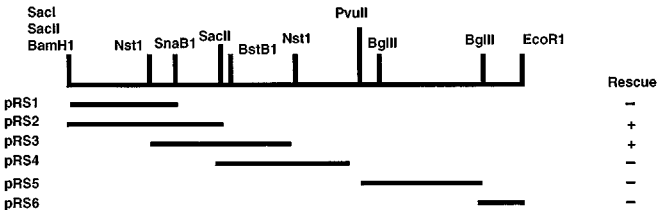

FIG. 4. **Mapping of the *dna2-1* mutation by marker rescue and DNA sequencing.** Six different fragments spanning the *DNA2* gene were cloned into pRS424 to give the respective plasmids: pRS1, 1.1-kb *Sac*I-*Sna*B1 fragment of the *dna2-1* gene; pRS2, 1.4-kb *Sac*II fragment; pRS3, 1.34-kb *Nst*I fragment; pRS4, 1.14-kb *Bst*B1-*Pvu*II fragment; pRS5, 1.1-kb *Bgl*II fragment; pRS6, 0.4-kb *Bgl*II-*Eco*RI fragment. These plasmids were used to transform *dna2-1*$_{ts}$. Transformants were selected by growth on uracil-deficient plates and replica plated, and the replicas were placed at 37 °C. The appearance of papillation after 2 days indicated recombination and hence marker rescue. The *Sna*BI-*Sac*II region of the *dna2-1*$_{ts}$ gene was amplified by PCR and cloned. Six different clones were prepared from six different PCR reactions to avoid being misled by mutations that might occur during PCR amplification. DNA sequencing revealed that each had not one, but two, base changes. One mutation changed proline 504 into serine (CCT to TCT) and is likely the *dna2-1* mutation, since the second base change is silent, leaving amino acid 426 as serine (AGC to AGT). The figure shows a diagram of the segments used to both functionally (marker rescue) and physically map the mutation. + indicates papillation after 2 days and constitutes marker rescue; − indicates lack of papillation. The mutation causing the *ts* phenotype falls between the *Sna*BI site and the *Sac*II site.

not able to completely remove contaminating proteins, preventing us from determining whether the helicase was intrinsic to or merely associated with the HA-Dna2p (2). As shown in Fig. 3, DNA helicase activity is observed with purified wild-type HA-Dna2K protein (Fig. 3, *lane 2*) but not with the mutant HA-Dna2E protein (Fig. 3, *lane 3*). Thus, DNA helicase activity is intrinsic to the Dna2 protein. (Interestingly, a structure-specific nuclease activity, which preferentially degrades a substrate with the configuration shown in Fig. 3 (2), is also present in these highly purified preparations (see below). The nuclease is not affected by the lysine to glutamate mutation, as expected, since ATP is not required for nuclease activity (2).)

The complementation of the *ts* mutant and the deletion mutant taken together demonstrate that the DNA-dependent ATPase and helicase activity of Dna2p is required for its essential role in DNA replication. It was therefore of interest to ask whether the *dna2-1*$_{ts}$ mutation affected the helicase domain. The site of the *dna2-1*$_{ts}$ mutation was mapped using a marker rescue technique we previously used to locate temperature-sensitive mutations of the *POL1* gene, encoding DNA polymerase α (Fig. 4) (7). DNA sequencing (see Fig. 4) revealed a single amino acid change of proline 504 to serine (CCT to TCT), placing the *dna2-1*$_{ts}$ mutation in the N-terminal portion of the protein, far from the essential helicase domain (amino acids 1070–1522). Thus, the Dna2p appears to be composed of at least two domains, both required *in vivo*. Nevertheless, intraallelic complementation of the *GAL10*-expressed ATPase mutant protein in the *dna2-1*$_{ts}$ strain did not occur (Fig. 1), suggesting that the functional domains of the Dna2 protein cannot act in trans *in vivo*.

Both sequence conservation and deletion analysis also support an important role for the N-terminal domain. Dna2p is similar over its entire length to the human ha3631 gene product, an open reading frame derived from DNA sequence (accession no. D42046), having 34% overall amino acid sequence identity and 55% similarity to Dna2p (2). The proline changed by the *dna2-1*$_{ts}$ mutation is conserved between the Dna2 protein and the ha3631 open reading frame and falls in an N-terminal 20-amino acid stretch that is 55% identical and 98% similar to the human ha3631 gene product. Such strong conservation suggests that the proline and surrounding motif are functionally important. Preparation of a series of deletions into the Dna2 protein was described previously (2). Deletion of only 25 amino acids at the C terminus results in a protein unable to complement the *dna2-1*$_{ts}$ mutation. Deletion of 105 amino acids from the N terminus leads to a protein that can complement both the *dna2-1*$_{ts}$ mutation and a *dna2*Δ strain and that is active as a helicase (2). However, deletion of an additional 25 amino acids inactivates the protein.

What is the role of the N terminus? The sequence does not contain motifs characteristic of any class of protein with known function. It may be essential for helicase activity. Alternatively, it may not contribute directly to the catalytic activity of the helicase but may rather serve as a site of protein/protein interactions. If Dna2 protein is oligomeric, as many helicases are, then association of monomers might be destabilized in the *dna2-1*$_{ts}$ protein. The partial dominant negative effect described above may suggest an oligomeric structure, in analogy with phage T7 gene 4 mutants (8).

It is also possible that the N-terminal domain is required for interaction with other replication proteins. Preliminary evidence suggests that Dna2 protein interacts with the product of another yeast replication gene, the *YKL510*/*RAD27* gene, a homolog of human FEN1 endonuclease, which is involved in processing of Okazaki pieces in the SV40 *in vitro* replication system (9–11). The YKL510/Rad27 nuclease copurifies with Dna2 helicase through all purification steps (Fig. 3).[2] Furthermore, a plasmid that overexpresses the YKL510 (*RAD27*) gene suppresses the *dna2-1*$_{ts}$ mutation but not *dna2*Δ.[2] Such high copy suppression is considered genetic evidence for interaction of the corresponding gene products *in vivo*. A deletion of the *RAD27* gene results in a strain with temperature-sensitive growth (10, 12).[2] Thus, the temperature-sensitive phenotype of the *dna2-1*$_{ts}$ strain may result from an inability of the mutant protein to interact with *RAD27* or an additional nuclease involved in Okazaki fragment processing. Even if the latter hypothesis is correct, it is likely that additional factors contribute to the temperature-sensitive phenotype of the *dna2-1*$_{ts}$ mutation, since *dna2-1*$_{ts}$ strains exhibit less DNA synthesis at 37 °C than *rad27*Δ strains (2, 10).

At this stage of characterization of Dna2p, it is hard to predict its precise mechanistic role in DNA replication. The 3′ to 5′ directionality might suggest that unwinding at a chromosomal fork may be coordinated with polymerization of the lead-

[2] M. E. Budd and J. L. Campbell, unpublished data.

ing strand. Given the complexity of the eukaryotic replication fork, the requirement for a 3′ to 5′ helicase does not exclude a role for additional helicases in yeast chromosomal DNA replication, including at least one that, like the prokaryotic primosomal helicases, has a 5′ to 3′ polarity.

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