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 strain defective in the DNA2 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 immun affinity-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 excision 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 trpl-289 ura3-1,2; BJ 5459, a ura3-52 trpl-1 lys2-801 leu2 trpl1 (his3 A1153 prbl16.6); J D52, a leu2-3,112 his3A200 trpl-52 lys1-801; J D53, a leu2-3,112 his3A200 trpl163 ura3-52 lys2-801; and MB1, a DNA2 trpl-52 ura3-52 leu2-3,112 his3A200 trpl163 lys2-801. In the plasmids used pJ Dgal2, which has the DNA2 gene (amino acids 1035-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred. pGal18-Dna2HA has the DNA2 gene (amino acids 105-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred. pGal18-Dna2HA has the DNA2 gene (amino acids 105-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred. pJ Dgal2, which has the DNA2 gene (amino acids 1035-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred. pGal18-Dna2HA has the DNA2 gene (amino acids 105-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred. pGal18-Dna2HA has the DNA2 gene (amino acids 105-1522) cloned into the EcoRI site of pJ Dgal 5′ and pJ Dgal-DNA2E has the DNA2 gene with codon 1080 changed to glutamate by site-directed mutagenesis and then cloned into the plasmid pJ Dgal at the EcoRI site. DNA sequencing verified that only one change had occurred.

Purification of Dna2p—HA-Dna2 protein was purified from BJ 5459 cells transformed with pGal18-Dna2HA or pJ Dgal-DNA2E. Transformed cells were grown in 2% synthetic raffinose media to 107 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, 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 polyethyleneglycol glycol 8000 was added to 6% to remove DNA. After 15 min,

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‡Theabbreviationsusedare: DTT, dithiothreitol; kb, kilobase pair(s); PCR, polymerase chain reaction.
The helicase domain of Dna2p contributed to its essential physiological role in mRNA transcription (3, 4). To investigate whether the helicase activity of the Rad3 protein is dispensable for its essential function in the completion of DNA replication, the rad3-1ts strain was then assessed. As shown in Fig. 1, dna2-1ts cells expressing the wild-type (DNA2K) and ATPase mutant (DNA2E) genes, pΔgal:DNA2K or pΔgal: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. 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, pΔgal:DNA2K or pΔgal: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.

**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 G23GK(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 dna2-1ts strain and a dna2Δ deletion strain was then assessed. As shown in Fig. 1, dna2-1ts 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 Dna2A/dna2Δ strain, used previously to demonstrate that Dna2A was essential (2), was transformed with both wild-type pΔgal:DNA2K and mutant pΔgal:DNA2E plasmids. The transformants were sporulated and tetrads analyzed. Transformants carrying pΔgal:DNA2K gave rise to viable spores in all tetrads, while pΔgal:DNA2E failed to complement dna2A spores, giving rise to 2 viable and 2 inviable spores in each tetrad. Interestingly, the dna2A strain transformed with pΔgal:DNA2K grew on glucose as well as on the inducing carbon source galactose. In contrast, the pΔgal:DNA2K plasmid complemented dna2A-1ts strains only after induction on galactose (Fig. 1). This suggests that when expression of the wild-type Dna2 protein is low, the dna2A-1ts protein may exhibit a dominant negative effect, perhaps by forming inactive heteroallelic dimers containing a mixture of wild-type and mutant protein.

The dna2A-1ts mutation is recessive in a heterozygous strain transformed with pΔgal:DNA2K grown with galactose, raising the level of wild-type Dna2 protein eliminates the possible dominant negative effect of dna2A-1ts 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 Pi, 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.

**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.
not able to completely remove contaminating proteins, prevent-
ing 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 sub-
strate 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 mu-
tant taken together demonstrate that the DNA-dependent
ATPase and helicase activity of Dna2p is required for its es-
cential role in DNA replication. It was therefore of interest to
ask whether the dna2-1ts mutation affected the helicase do-
main. The site of the dna2-1ts mutation was mapped using a
marker rescue technique we previously used to locate temper-
ature-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).
Thus, the Dna2p appears to be composed of at least two domains, both required in vivo. Nevertheless, an intracellular complementation of the GAL10-expressed ATPase
mutant protein in the dna2-1ts 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 sup-
port an important role for the N-terminal domain. Dna2p is
similar over its entire length to the human ha3631 gene prod-
uct, an open reading frame derived from DNA sequence (acces-
sion no. D42046), having 34% overall amino acid sequence
identity and 55% similarity to Dna2p (2). The proline changed
by the dna2-1ts mutation is conserved between the Dna2 pro-
tein 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 con-
servation 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 dna2-1ts mutation. Deletion of 105 amino acids
from the N terminus leads to a protein that can complement
both the dna2-1ts mutation and a dna21 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 inter-
actions. If Dna2p protein is oligomeric, as many helicases are, then association of monomers might be destabilized in the
dna2-1ts protein. The partial dominant negative effect de-
scribed 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 evi-
dence 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 Further-
more, a plasmid that overexpresses the YKL510 (RAD27) gene
suppresses the dna2-1ts mutation but not dna2a.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
dna2-1ts strain may result from an inability of the mutant
protein to interact with RAD27 or an additional nuclease in-
volved in Okazaki fragment processing. Even if the latter hy-
thesis is correct, it is likely that additional factors contribute
to the temperature-sensitive phenotype of the dna2-1ts mutation,
since dna2-1ts strains exhibit less DNA synthesis at 37°C than
rad27a 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 chro-
mosomal fork may be coordinated with polymerization of the lead-
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 primsomal helicases, has a 5' to 3' polarity.

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