Biochemical and Biological *in vivo* Functions of Dna2p in *Saccharomyces cerevisiae*

Thesis by

Wonchae Choe

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This thesis is dedicated to my parents,

Yongchin and Chinyoul Choe,

And my children, Hyunjune and Hyunjung Choe

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Abstract

We have characterized a temperature-sensitive yeast mutant, *dna2ts*, which is defective in DNA replication. DNA2 is essential and encodes a 172-kDa protein with a DNA helicase motif at its C-terminal portion. A homology search showed that Dna2p is conserved structurally among species. Even *Xenopus laevis Dna2* was able to complement an *S. cerevisiae dna2-1* mutant strain for growth at the nonpermissive temperature, suggesting that Dna2p is conserved also functionally. The site of the *dna2-1* mutation was mapped using a marker rescue technique and turned out proline 504 to serine, placing the *dna2-1* mutation in the N-terminal portion of the protein, suggesting that N-terminal portion of the protein is important for the activity of Dna2p. Recombinant ScDna2p was expressed in insect cells and purified. With the purified protein, we were able to demonstrate that Dna2p was a single-stranded DNA endonuclease/helicase and a single stranded DNA-dependent ATPase, suggesting that Dna2p has various biochemical functions.

We also found that Dna2 helicase-nuclease is a component of telomeric chromatin. Both chromatin immunoprecipitation and immunofluorescence showed that Dna2p associates with telomeres but not the bulk of chromosomal DNA in G1 phase. In S phase, there is a dramatic redistribution of Dna2p from the telomeres to sites throughout replicating chromosomes. Dna2p is again localized to telomeres in late S, where it remains through G2 and until the next S phase. Telomeric localization of Dna2p required Sir3p, since the amount of Dna2p found at telomeres by two different assays, one hybrid and ChIP, is severely reduced in strains lacking Sir3p. The Dna2p is also distributed throughout the nucleus in cells growing in the presence of DSB-inducing agents such as bleomycin. Finally, we show that Dna2p is functionally required for telomerase-dependent de novo telomere synthesis and also participates in telomere lengthening in mutants lacking telomerase, and that genetic instability due to *dna2* mutations lead to premature aging phenotype.

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Chapter 1: DNA replication, DNA repair and DNA recombination at telomere: An introduction

Chromosomal DNA replication

During S phase of the eukaryotic cell cycle, chromosomal DNA is replicated precisely once before mitosis by the carefully regulated initiation process. The initiation of DNA replication involves the assembly of the complex of the replication fork protein on the initiation locus (replication origin). The exact mechanism for initiation in eukaryotic cell is not clear, while in *S. cerevisiae* this initiation mechanism is very clear. Yeast origins consisting of a number of highly conserved 12 bp core origin are recognized and fired by a multiprotein origin recognition complex (orc)(Bielinsky and Gerbi, 2001; Takahashi and Masukata, 2001).

Once replication fork is formed, it has to carry out a number of functions while moving along the DNA. Ahead of replication fork, the two strands of DNA are unwound, requiring a temporal disruption of the normal chromatin structure. Torsional stress produced by the unwinding is relieved by topoisomerse (Badaracco et al., 1983). Since DNA polymerase can synthesize DNA only from 5' to 3', the leading strand is synthesized in a continuous direction, while the lagging strand in a discontinuous way, requiring additional lagging strand maturation machineries.

It involved several steps. First, after synthesis, RNA primers are removed, the gaps filled with DNA polymerase, and the fragments are ligated together. Replicated DNA is then reassembled into chromatin, which requires the reassembly of non-histone proteins

such as transcription factor, and reconstitution of chromatin modification and DNA methylation (Waga et al., 1994). The current view of DNA replication in eukaryotes predicts that pol α/primase synthesizes the first RNA/DNA primer on the leading strand and at each Okazaki fragment on the lagging strand. Then replication factor C (RF-C) binds to the 3'-OH end of the nascent DNA strand and loads proliferating cell nuclear antigen (PCNA), thereby displacing pol α (Ehrenhofer-Murray et al., 1999; Waga et al., 1994). The displacement of pol α occurs after 30 nt, likely by RF-C action. Next, pol δ is recruited and this event is called DNA polymerase switch. Beside its role in DNA replication, pol δ has been shown to be involved in DNA repair processes such as long patch base excision repair, nucleotide excision repair, and mismatch repair (Brown et al., 1993; Shivji et al., 1995). Pol δ is a heteromultimeric enzyme composed of one major catalytic subunit of 125 kDa and three small accessory subunits (Ng et al., 1993); by itself pol δ is a poorly processive enzyme due to its unstable interaction with the DNA substrate, but a physical interaction with the processivity factor PCNA leads to a catalytically competent processive holoenzyme (Ng et al., 1993). The consequence of such an interaction is a marked increase in the processivity (Brown and Campbell, 1993). Pol δ has intrinsic DNA polymerization and 3' → 5' exonucleolytic activities (Jin et al., 2001). In addition, a limited strand displacement activity has been described for the pol δ/PCNA/RF-C holoenzyme on a gapped M13 DNA template (Ng et al., 1993).

DNA repair and recombination

As a major defense against environmental damage to cells, DNA repair is present in all organisms examined including bacteria, yeast, Drosophila, fish, amphibians, rodents and

humans. DNA repair is involved in processes that minimize cell killing, mutations, replication errors, persistence of DNA damage and genomic instability. Abnormalities in these processes have been implicated in cancer and aging.

The most important DNA repair pathway is nucleotide excision repair that fixes the majority of bulky lesions in DNA. These lesions include UV induced photoproducts, and bulky adducts such as those derived from cisplatin and 4-nitroquinoline oxide. Understanding of the enzymology was previously based on knowledge from work done in E.coli, but now the molecular events are being characterized in human cells. Nucleotide excision repair involves recognition, incision, degradation, polymerization, and finally, ligation. The recognition steps involve the ERCC1, RPA products followed by the interaction with the TFIIH transcription factor having bending activity on damaged lesions. This factor contains the repair genes XPB and XPD and thus represents a direct molecular link between DNA repair and transcription (Habraken et al., 1993; O'Donovan et al., 1994). A dual incision event is accomplished by the ERCC1 and XPG products, and this is followed by polymerization and ligation (Tomkinson et al., 1993). Double strand break arise in DNA as a result of ionizing radiation and radiomimetic chemicals, and in mammalian cells during the VDJ chain recombination event. Repair of double strand break is carried out by homologous recombination pathway including RAD51 AND RAD52 or nonhomologous recombination pathway (NHEJ).

Telomere replication

The DNA molecules in eukaryotic chromosomes are linear; i.e., have two ends. The

DNA molecule of a typical chromosome contains a linear array of genes (encoding proteins and RNAs) interspersed with much of non-coding DNA. Included in the non-coding DNA are long stretches that make up the centromere and long stretches at the ends of the chromosome, the telomeres. As in most organisms, *S.cerevisiae* telomeres consist of multiple copies of the simple repeated DNA (Berman et al., 1986; DuBois et al., 2000). Each end of each chromosome bear about 300+75bp c2-3a (ca) 1-6/(tg) 1-6tg2-3 DNA, commonly abbreviated as C1-3A/TG1-3 (Shampay, 1984).

In addition to that, *S.cerevisiae* telomeres carry middle repetitive DNA sequences called telomere-associated repeats (TA). The y' subtelomeric repeat is found 0 to 4 tandom copies at individual telomeres. The chromosome distribution of Y' varies considerably strain to strain. The second class of TA is X. X is quite variable in size but exists in most telomeres (Shampay, 1984).

The C1-3A repeats are in a non-nucleosomal chromatin structure called the telosome while X and Y are assembled in nucleosome (Dubrana et al., 2001). Nonetheless, nucleosomes at subtelomere are different than any other nucleosome because of the hypoacethylation and hypodam methylation. This observation could be due to telomere position effect, which is the transcriptional silencing at telomeres (Kennedy et al., 1997; Tsukamoto et al., 1997).

End replication problem

Wherever the replication fork of a strand is moving towards the 3' end, the newly synthesized DNA begins as Okazaki fragments. This continues until close to the end of the chromosome. However, the molecular requirements of the process are such that 5'

end of each newly synthesized strand cannot be completed because of the gap left by removal of the last RNA primer. Thus each of the daughter chromosomes will have a shortened telomere. It is estimated that human telomeres lose about 100 base pairs from their telomeric DNA at each mitosis. This represents about 16 TTAGGG repeats. At this rate, after 125 mitotic divisions, the telomeres would be completely gone. This paradox is called end replication problem (Ohki et al., 2001; Zakian, 1996). This shortening problem can be counteracted by a well-established pathway in which telomerase, a reverse transcriptase, uses its associated RNA as a template to extend the 3' end (Blackburn, 1992; Dubrana et al., 2001; McEachern et al., 2000; Nugent and Lundblad, 1998). Alternatively, homologous recombination, in which DNA polymerase uses another telomere as a template to extend the 3' end, can be used to solve the same problem, which is described later in this chapter. Both mechanisms can use conventional repair synthesis to fill in the lagging strand.

Recombination at telomere

The first hint of a recombination pathway for telomere maintenance came from the continuously growing, unicellular eukaryote *Saccharomyces cerevisiae*. Early work characterizing telomeric repeats in this yeast led to the formulation of two models of telomere-length maintenance. One model proposed the existence of an enzyme that could add telomeric repeats *de novo* (now known to be telomerase). The other was based on a mechanism through which a recombination of telomeric sequences replenishes telomeric repeats (Le et al., 1999; Pluta and Zakian, 1989). The latter mechanism only became apparent on examination of yeast cells lacking a gene required for the

telomerase pathway. Because of the inability of conventional cellular DNA polymerases to replicate the very end of a linear molecule, telomeres shorten with each round of cell division in the strain lacking telomerases. If unattended, this shortening leads to chromosome instability and, ultimately, cell death. Whereas most of these cells showed evidence of telomere shortening and died, a small subset survived and ultimately thrived. The generation and stability of survivors was dependent on the function of Rad52p, an essential component of the homologous recombination pathway. The long and heterogeneous telomeres of survivors (like those generated by ALT), combined with Rad52p dependence, are consistent with a gene-conversion event initiating within telomeric sequences (Le et al., 1999; Tsukamoto et al., 1997). There are two types of recombination at telomeres to maintain telomeres in a strain lacking telomerases. Type I telomere recombination is dependent on Rad51 pathway. Type II telomere recombination is dependent on Rad50 pathway. Usually Type I comes early and Type II dominates later because Type II grows faster than Type I survivors.

Capping at a chromosomal end

Does a cell distinguish a double strand break damage DNA from the end of the chromosome? It is a very interesting question because the double strand break damaged DNA and telomere DNA share the same physical DNA structure with discontinuous end so that a cell would treat telomere as another double strand break damages. The answer to the above question is probably yes; otherwise, a cell cannot survive due to genetic instability caused by inappropriate double strand repair.

As mentioned about the structure of telomere, its repetitive sequence is bound by

protein complex to protect its ends from degradation and fusion and also to promote telomere replication (Shore, 1998; Zakian, 1996), but the mechanism by which nature recognize telomeres from the double strand break damages to protect telomeres is not known, yet. However, a lot of evidence showing this protection is emerging. It is reasonable to exclude double strand break damage repair complex from telomere not to mix telomere and double strand break. But this is not the case.

In yeast, as in mammals, several complexes directly involved in nonhomologous end joining (NHEJ) are telomere-bound and affect telomere length maintenance. Yeast ku protein involved directly in double strand break repair associate subtelomere and telomeric repeats, based on chromatin immunoprecipitation experiment results and immunofluorescence data (Gravel et al., 1998; Martin et al., 1999). Also strain-lacking yku dimmer shows short telomere and reduced telomere silencing (Gravel et al., 1998; Tsukamoto et al., 1997). Consistently, loss of either subunit is lethal when combined with mutations in telomerase (*EST2*) or in Cdc13p, a single-strand binding protein that helps protect the C-rich telomeric strand from degradation (Polotnianka et al., 1998).

Telomeres are affected not only by double strand break damage repair proteins, but also by DNA damage checkpoint proteins. Deletion of *MEC3*, which is a budding yeast checkpoint gene downstream of *RAD17*, induces the lengthening of telomeres and counteracts the derepression of telomeric silencing provoked by loss of *SET1*, a yeast member of the *trithorax* gene family.

In yeast, recent results suggest that a critical shortness must be reached to trigger telomerase action (Marcand et al., 1999). This result supports the suggestion made by Blackburn (Blackburn, 2000) in a recent review that telomeres switch between two states, in which the structure can be either 'accesible' or 'inaccesible' to the action of the

telomerase or other enzymes. In its open state, a telomere may mimic a free DNA end, which will elicit the action of other enzymes. If the original telomeric structure cannot be restored to 'inaccessible' state, an open telomere may be the recognized as double strand break, provoking cell cycle arrest. If so, which molecule plays a capping role at telomeres? In a review made by Gasser (Dubrana et al., 2001), possible capping molecules are mentioned based on the assumption that mutation in a capping molecule would expose it readily to telomerase, leading to longer telomere. They are Rap1, Rif1, Rif2, Mec3, and Stn1. Taken together, capping mechanism must be a key to understand how a cell distinguishes telomere from double strand break DNA, even though capping mechanism is not proved fully.

What is *DNA2* helicase?

Its biochemical activity

DNA2 gene was cloned in a screen for the mutants defective in DNA replication in vitro (Budd and Campbell, 1995). However, fluorescence-activated cell sorting analysis showed that DNA2-1 mutant actually synthesized full 2c content at 37°C as well as at 25°C (Budd and Campbell, 1995). But the bulk of DNA synthesized at 37 °C in the mutant was not intact. It turned out to be fragmented based on the result of the sucrose gradient analysis, suggesting that although there is extensive DNA replication; its joining process is somehow inhibited (Budd and Campbell, 1995).

As imagined from its seven helicase domains, it has strong helicase activity and single stranded DNA dependent ATPase (Budd and Campbell, 1995). Recently it has been shown to have a strong single-stranded DNA specific endonuclease. Both of helicase

and endonuclease were essential to cellular viability (Budd et al., 2000; Formosa and Nitiss, 1999; Lee et al., 2000).

Different from other DNA replication mutants, Dna2 mutation causes an increase in sensitivity to X-rays, and methylmethane sulfonate both in haploids and in homozygous diploids (Budd and Campbell, 2000a; Budd and Campbell, 2000b). The X-ray sensitivity of dna2 mutants is suppressed by overexpression of a 5' to 3' exonuclease, the yeast FEN-1 structure-specific nuclease, encoded by the RAD27 gene, which also suppresses the growth defect of dna2-ts mutants (Budd and Campbell, 2000b). It suggested that DNA2 might be involved in double-strand break repair only, or to repair the double strand break during the cell cycle, it requires the full replication machineries including the leading and lagging strand synthesis machineries.

Its related proteins and lethality

Genes homologous to *S.cerevisiae* DNA2 have been identified in human, *S pombe*, *Xenopus laevis*, and *C. elegance* (Budd et al., 2000; Liu et al., 2000). Its high homology and its role at the replisome suggested that DNA2 is essential gene again. Recent homology search showed that Dna2 belongs to RecB class of helicase/ nuclease protein. Most nuclease domain of dna2 protein was localized to N terminal half, corresponding to the active site of RecB nuclease/helicase (Budd et al., 2000) .

Its genetic and physical interaction

Fen-1 is not only 5'-3'exonuclease but also endonuclease that functions on 5' single strand flap structure adjacent to a duplex region. Human Fen-1 is known to function for Okazaki fragment maturation in SV40 in vitro system (Waga et al., 1994). Rad27 is

yeast homolog of FEN1. Surprisingly, rad27 null mutation gives its phenotype, leading to a high level of mutagenesis. For example, rad27 deletion mutant give rise to high frequency of duplication at repetitive sequences due to unstable Okazaki fragment maturation. When the long CAG track was used for lagging strand substrate, most mutants involved in lagging strand synthesis including rad27, cdc9, and pri1, showed increased instability on this track. Even though Dna2 mutant does not show high frequency of duplication, there are strong genetic and physical interactions between DNA2 and rad27. Overexpression of *RAD27* complements *dna2–1* strains and overproduction of *DNA2* suppresses the temperature-sensitive growth defect of *rad27*Δ strains. *rad27*Δ *dna2–1* strains are inviable. Biochemical studies showed that the affinity-purified Dna2 preparations contained FEN-1, and that FEN-1 and Dna2 were co-immunoprecipitated from yeast extracts. This genetic and biochemical evidence suggest that Dna2, like FEN-1, functions in Okazaki fragment maturation.

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Chapter 2: Biochemical Characterization of Dna2p in *Saccharomyces Cerevisiae*

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DNA2 Encodes a DNA Helicase Essential for Replication of Eukaryotic Chromosomes

Martin E. Budd , **Won-Chae Choe** , Judith L. Campbell

Braun Laboratories, California Institute of Technology, Pasadena, California 91125

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

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Martin E. Budd, Won-Chae Choe, and Judith L. Campbell‡

From Braun Laboratories, California Institute of Technology, Pasadena, California 91125

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 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 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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‡ To whom correspondence should be addressed: Braun Laboratories, 147-75, California Institute of Technology, Pasadena, CA 91125. Tel.: 818-395-6053; Fax: 818-405-9452; E-mail: campbellj@starbase1.caltech.edu.

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

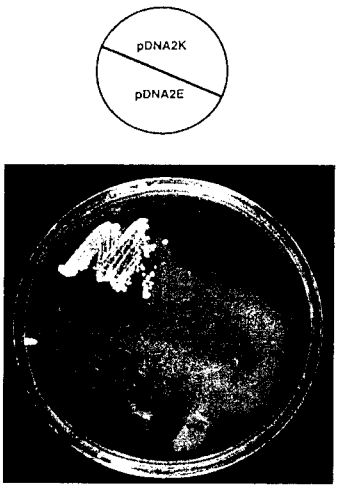

FIG. 1. **Lack of complementation of *dna2-1* by the Dna2E mutant protein.** 3X154 9A *dna2-1 ura3* was transformed with the plasmids expressing the wild-type (*DNA2K*) and ATPase mutant (*DNA2E*) genes, pJDgal:DNA2K or pJDgal:DNA2E. Ura$^+$ transformants were selected at 23 °C. Both plasmids yielded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

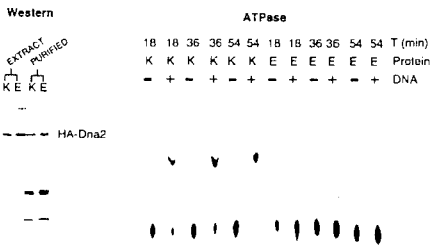

FIG. 2. **Mutation of the conserved ATP-binding site eliminates the DNA-dependent ATPase activity of Dna2p.** Protein (0.2 mg of the 0.2 M hydroxyapatite wash prepared as described under "Materials and Methods") was mixed with 20 μg of 12CA5 monoclonal antibody for 1 h at 0 °C. Twenty microliters of 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$_4$GK(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

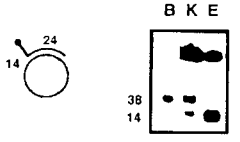

FIG. 3. **Mutation of the conserved ATP-binding site eliminates the DNA helicase activity of Dna2p.** The wild-type and mutant proteins eluted from hydroxyapatite were purified further by immunoaffinity chromatography (see "Materials and Methods" and Ref. 2). Purified protein (10 ng) was assayed for 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.

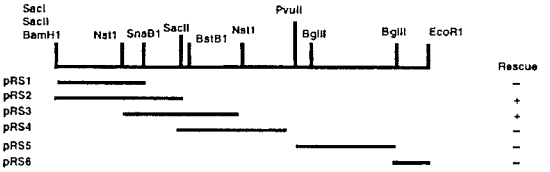

FIG. 4. **Mapping of the *dna2-1* mutation by marker rescue and DNA sequencing.** Six different fragments spanning the *DNA2* gene were cloned into pRS424 to give the respective plasmids: pRS1, 1.1-kb *Sac*I-*Sna*B1 fragment of the *dna2-1* gene; pRS2, 1.4-kb *Sac*II fragment; pRS3, 1.34-kb *Nsi*I fragment; 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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Identification of the Xenopus laevis Homolog of Saccharomyces cerevisiae DNA2 and Its Role in DNA Replication*

Qingquan Liu, **Won-chae Choe**, and Judith L. Campbell

From the Braun Laboratory, California Institute of Technology, Pasadena, California 91125

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Identification of the *Xenopus laevis* Homolog of *Saccharomyces cerevisiae DNA2* and Its Role in DNA Replication*

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Qingquan Liu, Won-chae Choe, and Judith L. Campbell‡

From the Braun Laboratory, California Institute of Technology, Pasadena, California 91125

The *DNA2* gene of *Saccharomyces cerevisiae* is essential for growth and appears to be required for a late stage of chromosomal DNA replication. *S. cerevisiae* Dna2p (ScDna2p) is a DNA helicase and also a nuclease. We have cloned and sequenced the homologous gene from *Xenopus* (*Xenopus* Dna2). *Xenopus* Dna2p (XDna2p) is 32% identical to ScDna2p, and the similarity extends over the entire length, including but not limited to the five conserved helicase motifs. XDna2p is even more closely related (60% identical) to a partial human cDNA. The *Xenopus* Dna2 (XDna2) gene was able to complement an *S. cerevisiae dna2-1* mutant strain for growth at the nonpermissive temperature, suggesting that XDna2p is a functional as well as a structural homolog of the yeast protein. Recombinant XDna2p was expressed in insect cells and purified. Like the ScDna2p purified from yeast, it is a single-stranded DNA endonuclease and a DNA-dependent ATPase, suggesting that both of these activities are part of the essential function of Dna2p. However, unlike ScDna2p from yeast, recombinant XDna2p showed no DNA helicase activity. When XDna2 was immunodepleted from interphase egg extracts, chromosomal DNA replication was almost completely inhibited. From the size of the residually synthesized DNA from the XDna2-depleted egg extracts, it seems that initiation of DNA replication may be impaired. This interpretation is also supported by the normal DNA replication of M13 single-stranded DNA in the XDna2-depleted egg extracts.

Although the mechanism of DNA replication is very well understood in virus, phage, and bacterial systems, knowledge about how the replication of chromosomal DNA in eukaryotic cells is initiated, elongated, and terminated and how the fidelity of replication is achieved is still limited (1). Identification of additional proteins involved in chromosomal DNA replication in eukaryotes should afford further insight into these processes. The *DNA2* gene from *Saccharomyces cerevisiae* (Sc*DNA2*)[1] is essential for viability. Sc*DNA2* encodes a protein of 170 kDa with characteristic DNA helicase motifs. Highly purified ScDna2p isolated from *S. cerevisiae* has intrinsic DNA-dependent ATPase and 3′ to 5′ DNA helicase activity specific for forked substrates. A single-stranded DNA endonuclease activity also copurifies with ScDna2p from yeast (2, 3). Purification of recombinant ScDna2p from insect cells demonstrated that the single-stranded DNA endonuclease activity was intrinsic to ScDna2p; and, furthermore, an additional comparatively minor nuclease that was ATP-dependent and specific for double-stranded DNA was found in the highly purified preparations (4). Curiously, although the recombinant ScDna2p purified from insect cells had DNA-dependent ATPase, it did not show the helicase activity found in ScDna2p purified from *S. cerevisiae*. This discrepancy may indicate that either posttranslational modification or other protein(s) are required for the helicase activity of ScDna2p (4).

Several lines of evidence point to a role for the Dna2p in DNA replication. Under restrictive conditions, temperature-sensitive Sc*dna2-1* mutants do not synthesize full-length chromosome-sized DNA and arrest in the cell cycle with a dumbbell-shaped morphology in a Mec1-dependent manner. Permeabilized cells and yeast nuclear extracts capable of semiconservatively replicating supercoiled plasmids have a reduced replication efficiency in the absence of ScDna2p (6, 7). The *Schizosaccharomyces pombe DNA2* gene has also been identified and is essential for growth. In high copy number, the *S. pombe DNA2* gene suppresses the defect in the essential function of *cdc24*. Although the specific role of Cdc24p is unknown, *CDC24* is an essential gene implicated in DNA replication, since mutants arrest in G_2 with rapid loss of viability and chromosomal breakage at the nonpermissive temperature (8). Thus, the function of Dna2p may also be conserved in other species.

But what does Dna2p do during DNA replication? Clues may be offered by the genes and gene products with which Sc*DNA2* interacts. Human Fen1 protein is a nuclease required for the processing of Okazaki fragments in the Simian virus 40 *in vitro* replication system (9–11). Genetic evidence suggests that ScDna2p and ScFen1p either interact or have partially overlapping functions. First, overproduction of ScFen1p, encoded by *RAD27*, suppresses the temperature-sensitive growth of a *dna2-1* mutant, and similarly overproduction of ScDna2p suppresses the temperature-sensitive growth of a *rad27*Δ mutant. Second, *dna2-1* and *rad27*Δ alleles are synthetically lethal. Third, certain *rad27* mutants require overproduction of the G_1 cyclins for growth, and overproduction of ScDna2p suppresses this requirement (12). Since ScDna2p has a nuclease activity similar to ScFen1p, it is possible that they can serve the same function in replication, accounting for the genetic observations. However, ScDna2p and ScFen1p also coimmunoprecipitate (13). Thus, they may actually have independent functions in a similar stage of replication, in which case the genetic data could be explained by their existing in a complex that is stable only in the presence of both proteins. Regardless of the molec-

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‡To whom correspondence should be addressed. Tel.: 626-395-6053; Fax: 626-405-9452; E-mail: jcampbel@cco.caltech.edu.

[1] The abbreviations used are: Sc*DNA2* and Sc*dna2*, *S. cerevisiae DNA2* and *dna2*, respectively; ScDna2p, *S. cerevisiae* Dna2p; XDna2p, *X. laevis* Dna2p; XDna2, *X. laevis* Dna2; HDna2p, human Dna2p; ADna2p, *A. thaliana* Dna2p; CDna2p, *C. elegans* Dna2p; nt, nucleotide(s); ScFen1, *S. cerevisiae* Fen1; NTER, N-terminal portion of XDna2p; PCR, polymerase chain reaction; DTT, dithiothreitol; 6-DMAP, 6-dimethylaminopurine.

ular details, the *DNA2/RAD27* interactions suggest that one possible role for ScDna2p lies in Okazaki fragment metabolism, and ScDna2p collaborates with or substitutes for ScFen1 in the processing of Okazaki fragments. This point of view was further suggested by the data from a recent paper in which it was shown that both overexpression of ScDna2 and deletion of ScFen1 yielded single-stranded DNA regions on telomeric DNA, and the accumulated single-stranded DNA was the templating strand for lagging-strand synthesis (14).

ScDNA2 was found to genetically interact with *POL1* and *CTF4*, which encode a DNA polymerase subunit and an associated protein (15–17). ScDna2p purified from *S. cerevisiae* contains Ctf4p.[2] Therefore, it seems possible that ScDna2p somehow interacts with DNA polymerase α, the DNA polymerase involved in the initiation of DNA synthesis both at origins of replication and at the site of each new Okazaki fragment. Recent, unpublished yeast studies also suggest a role for *DNA2* in the early events in S phase. *dna2-1* and *mcm10* are synthetically lethal in yeast.[3] Mcm10 is thought to be involved in initiation (18). Mutation of *mcm10* suppresses mutations in the better known *mcm2–7* genes.[4] A role for Dna2 in a late stage of DNA replication is favored, however, since despite the fact that *dna2* mutants fail to synthesize high molecular weight DNA at the restrictive temperature, *dna2* mutant haploid strains arrest with an apparent 2C DNA content (3, 5).

In order to establish the stage of replication in which Dna2p participates, we wanted to take advantage of the well established *in vitro* DNA replication system from *Xenopus*. In order to do so, we have isolated the *Xenopus* homolog (XDna2) of Sc*DNA2*, whose characterization we report here. The XDna2 gene is probably a functional homolog of Sc*DNA2*, since XDna2 efficiently complements an Sc*dna2* mutant for growth at the nonpermissive temperature. Like ScDna2p purified from insect cells, purified recombinant XDna2p has DNA-dependent ATPase and single-stranded DNA-specific endonuclease but no detectable DNA helicase activity. Furthermore, chromosomal DNA replication was almost completely inhibited in XDna2-depleted interphase egg extracts.

EXPERIMENTAL PROCEDURES

Materials—All oligonucleotides were synthesized by a California Institute of Technology facility. Radioactive materials and unlabeled ATP were from Amersham Pharmacia Biotech.

Cloning of the Xenopus Dna2 Gene—A *DNA2* probe for screening a *Xenopus* oocyte cDNA library was prepared by PCR using degenerate primers complementary to two motifs conserved among human, *Caenorhabditis elegans*, and *S. cerevisiae* Dna2 proteins (motifs 2 and 3; Fig. 1): primer A, ATCGAATTCACICA(T/C)I(C/G)IGCIGTIGA(T/C)AA-(T/C)ATI(T/C)T; primer B, AGGAAGCTTIGCIAC(A/G)TTIA(A/C/G)IC-(G/T)IC(G/T). ine extra nucleotides, underlined in each primer, provide restriction sites for *Eco*RI or *Hin*dIII. *Xenopus* oocyte cDNA (gift of Dr. William Dunphy (California Institute of Technology, Pasadena, CA)) was used as the substrate for PCR. The PCR product was electrophoresed on an agarose gel, and the expected 150-base pair band was isolated, digested with *Eco*RI/*Hin*dIII, and cloned into plasmid LITMUS39 (New England Biolabs) to give plasmid pEHPROBE. The *Eco*RI/*Hin*dIII fragment of pEHPROBE was radiolabeled and used to probe a *Xenopus* oocyte cDNA library (gift of Dr. William Dunphy) (19). Two clones were isolated, and the larger appeared, after DNA sequencing, to comprise a complete cDNA. The smaller clone was a partial cDNA of the same gene. The plasmid containing the full-length cDNA was called pXDna2.

Preparation of Polyclonal Antibodies against XDna2—Two polyclonal antibodies from rabbits were prepared, one against a C-terminal 12-amino acid peptide of XDna2p and the other against the N-terminal 712 amino acids of XDna2p. To obtain the latter immunogen, the XDna2 initiation codon was converted to an *Nde*I restriction site by PCR. (The primer containing the *Nde*I site was GCGGCAGCCATATGGAACCAGTGAGTGCTGAGT.) The second primer (GAGCTTTAGATTTCTGGTGTA) was complementary to a region between the internal *Bam*HI and *Eco*RI sites of XDna2. The PCR product was digested with *Nde*I and *Bam*HI and was cloned into the *Nde*I and *Bam*HI sites of pET-28a(+) (Novagen) to give pETNB, which contains the DNA fragment from the initiation codon to the *Bam*HI site of XDna2 fused to a six-histidine (His_6) tag at the start codon. The *Bam*HI/*Xho*I fragment of the XDna2 gene was cloned into the *Bam*HI and *Xho*I sites of pETNB to give plasmid pETNX; the *Nde*I/*Eco*RI fragment of pETNX was cloned into pET-28(+) to give plasmid pET712aa, which contains a DNA fragment corresponding to the first 712 amino acids of the XDna2 gene, plus the His_6 tag. pET712aa was introduced into *E. coli* strain BL21(DE3) (Novagen), and the expressed protein was purified using histidine binding resin (Novagen). The purified protein was loaded onto a preparative SDS-polyacrylamide gel, the gel was stained with Coomassie Brilliant Blue, the protein band was cut out and destained, and the destained gel slices were sent to BAbCo for injection into rabbits.

Expression of XDna2p in Insect Sf9 Cells—The Bac to Bac Baculovirus Expression system (Life Technologies, Inc.) was used to express the whole XDna2 gene. The XDna2 gene was cloned into pFASTBacHTa in the following procedure. The small *Ehe*I/*Eco*RI fragment in the polylinker site of pFASTBacHTa was replaced by a linker containing a *Nde*I site to give plasmid pFASTNdeI; the XDna2 C-terminal fragment from the *Eco*RI to the *Mun*I site was inserted into the *Eco*RI site of pET712aa to give plasmid pETXDna2, containing the whole XDna2 gene; the *Nde*I/*Xho*I fragment containing the XDna2 gene from pETX-Dna2 was inserted into *Nde*I/*Xho*I sites of pFASTNdeI to give pFASTX-Dna2. pFASTXDna2 was the donor plasmid for preparation of the recombinant bacmid. The expressed XDna2p contains a six-residue histidine tag at the N terminus of the protein and is designated His_6-XDna2p.

Purification of Recombinant XDna2p Expressed in Insect Sf9 Cells—50 ml of Sf9 cells infected with recombinant baculovirus at a multiplicity of infection of 10 was harvested 50 h after infection. Cells were washed with cold Tris-buffered saline buffer and resuspended into 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and 5 mM EGTA). Cells were broken using sonication. Cell extracts were spun at 12,000 rpm for 10 min at 4 °C. The supernatant was incubated with 100 μl of Ni^{2+}/NTA resin (Novagen) equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole) for 1 h at 4 °C. The resins were washed with 4 ml of binding buffer and subsequently 4 ml of washing buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 60 mM imidazole). XDna2p was eluted with 150 μl of buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 300 mM imidazole, and 10% glycerol). All of the buffers except the elution buffer contain protease inhibitors: phenylmethylsulfonyl fluoride (1 mM), pepstatin (2 μg/ml), leupeptin (1 μg/ml), and benzamidine (2 mM).

Complementation of S. cerevisiae dna2-1 by the XDna2 Gene—The *Bam*HI/*Sal*I fragment containing the C-terminal 2.8 kilobase pairs of the XDna2 gene from pETXDna2 was cloned into *Bam*HI/*Sal*I sites of pSEY18-GAL (20) to give plasmid pSEYBS. To clone the remaining N-terminal 0.4 kilobase pairs into *Bam*HI site of pSEYBS, PCR was performed (pETXDna2 was the substrate, and the sequences of two primers were CGCGGATCCAT GGAACCAGTGAGTGCTGAGTGCC and GAGCTTTAGATTTCTGGTGTA), and the PCR product was digested with *Bam*HI and cloned into pSEYBS. The resulting plasmid was called pGALXDna2, which contains the entire XDna2 gene under the control of a yeast *GAL* promoter. Strain 3×154-9 A*dna2-1 gal1 trp1–289 ura3–1* was transformed with pGALXDna2 at 23 °C, and complementation was tested by analysis of growth on galactose-raffinose plates at 37 °C.

Preparation of Radiolabeled Substrates for DNA Helicase and Nuclease Assay—A 44-mer oligodeoxynucleotide (AGCTCTTGATCGTAGACGTTGTAAAACGACGGCCAGTGCCAAGC) was labeled at the 5′-end using polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligonucleotide (2.5 pmol) was incubated with 2 μg of M13mp18 single-stranded DNA in 20 μl of annealing buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) at 65 °C for 5 min. The sample was slowly cooled to room temperature to anneal the 30 M13-complementary bases at the 3′-end of the oligonucleotide with M13 DNA. 80 μl of annealing buffer was added, and unincorporated [γ-^{32}P]ATP and unannealed oligonucleotide were removed by gel filtration using Sepharose CL-4B (Sigma). 3 μl was used for nuclease or helicase assays. For preparation of 3′-labeled substrate, the 44-mer was annealed with M13 DNA as described above and then labeled using Klenow enzyme and [α-^{32}P]dTTP. Annealing buffer was added to the 3′-labeled substrate to 100 μl. Gel filtration removed unincorporated [α-^{32}P]dTTP. The filtrate (3 μl) was used directly for the nuclease assay.

[2] M. E. Budd and J. L. Campbell, unpublished results.
[3] A. Sugino, personal communication.
[4] B. K. Tye, personal communication.

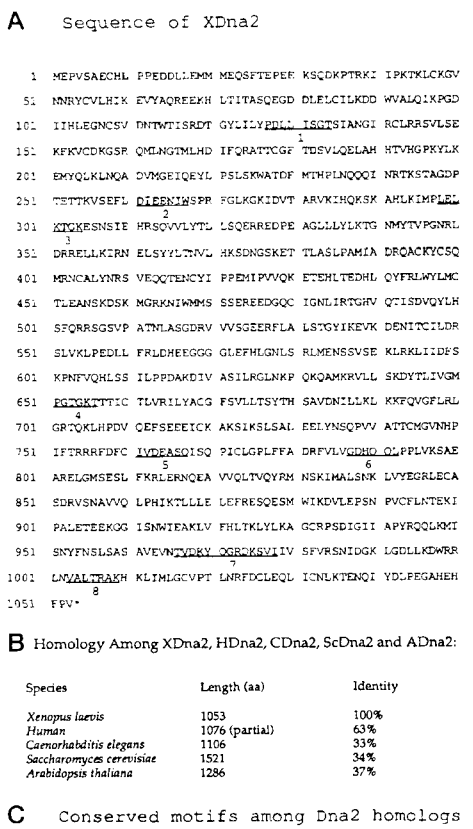

FIG. 1. **The *DNA2* gene is conserved from yeast to human.** *A*, the sequence of the cloned XDna2 gene is shown. XDna2p has 1053 amino acids. Eight highly conserved motifs are *underlined*. Motif 1 contains the proline residue that corresponds to the mutated proline in *Scdna2-1*. Motifs 2 and 3 are the two conserved motifs used to design the two primers for PCR to prepare the probe to isolate the XDna2 gene. Motifs 4–8 are the five conserved helicase motifs. Motifs 4 and 5 are the Walker A box and Walker B box, respectively. *B*, percentages of identical sequences among Dna2 proteins from different species. *C*, the five most highly conserved regions in XDna2p, HDna2p, ScDna2p, ADna2p, and CDna2p. The *underlined sequences* correspond to motifs 1–8 in *A*. The *numbers* shown *before* the sequences are the numbers of amino acids that exist before the first residues of the sequences shown. There are no numbers shown for HDna2p, because the cDNA sequence for human Dna2 is partial.

Nuclease Assay—Purified XDna2p was incubated with substrate (0.075 pmol of oligonucleotide) in 20 μl of reaction buffer (40 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 4 mM ATP, 2.5 mM DTT, and 5% glycerol) at 37 °C for 30 min. Then 10 μl of sequencing gel loading buffer was added. Samples were boiled for 3 min and loaded onto a 20% sequencing gel. The gel was run for 105 min at 20 watts. Gels were then dried, and results were analyzed using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

ATPase Assay—Purified protein (XDna2p or mutant) was incubated in 20 μl of reaction buffer containing 40 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 2 μM ATP, 2.5 mM DTT, 20 nM [α-^{32}P]ATP (3000 Ci/mmol), 0.1 mg/ml bovine serum albumin, 1 μg of poly(dA), and 5% glycerol at 37 °C for 1 h. Reaction mix (2 μl) was spotted onto a polyethyleneimine-cellulose plate (J. T. Baker Inc.), which was then developed in 0.5 M LiCl, 1.0 M formic acid solution. The results were analyzed using a PhosphorImager.

Preparation of Egg Extracts—Xenopus cytostatic factor-arrested and interphase egg extracts were prepared from unactivated eggs as described by Carpenter *et al.* (21).

Immunodepletion—Antibody in antiserum against the N-terminal 712 amino acids of XDna2 was affinity-purified using beads coupled with XDna2. 20 μl of protein A beads (Bio-Rad) was incubated with 40 μg of either affinity-purified anti-XDna2 antibody or a control rabbit anti-mouse antibody (Cappel) for 1 h at room temperature and then washed with 1× HBS buffer (25 mM K-Hepes, 8 g/liter NaCl, 0.2 g/liter KCl, pH 7.5). 20 μl of interphase egg extracts was incubated with 10 μl of beads from above for 45 min at 4 °C. The supernatant was recovered and incubated with a fresh aliquot of beads (10 μl) for 45 min at 4 °C. The supernatant was recovered again.

DNA Replication Assay—10 μl of normal interphase extract (*lanes 1* and *2*) or 6-dimethylaminopurine (6-DMAP)-treated extract (*lanes 3* and *4*) was incubated with 0.5 μl of [α-^{32}P]dATP and sperm nuclei (1000 nuclei/μl final concentration) for 1 h at room temperature. Subsequently, the reaction was stopped by the addition of 10 μl of 80 mM Tris-HCl (pH 8.0), 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, and 0.2% bromphenol blue. Proteinase K was added to digest the protein in the mixture. The DNA samples were loaded onto a 1% agarose gel and electrophoresed. The gel was dried and analyzed by PhosphorImager analysis.

RESULTS

Cloning of the XDna2 Gene—A data base search for protein sequences similar to Sc*Dna2* yielded a partial cDNA sequence from human (accession number KIAA0083) and a complete cDNA sequence from *C. elegans* (accession number Z50070). Regions conserved among these three proteins occur throughout the sequence, both in the C-terminal helicase domain and also in the N-terminal domain, which may encode the single-stranded DNA nuclease activity of Sc*DNA2*. Two conserved motifs in the N-terminal domain were used to design degenerate PCR primers to amplify a probe from *Xenopus* oocyte cDNA, which was then used to screen a *Xenopus* oocyte cDNA library. Two clones were identified, and the sequence of the plasmid with the longest insert was determined. The sequence is similar to Sc*DNA2* over its entire length and appears to be complete based on the existence of multiple stop codons in all three reading frames upstream of the proposed start codon (Fig. 1). The second clone represented a partial cDNA of XDna2. The protein deduced from the XDna2 sequence is 1053 amino acids, which is much shorter than ScDna2p (1522 amino acids). The missing amino acids form a continuous block at the N terminus of ScDna2p. This region does not appear to be essential for

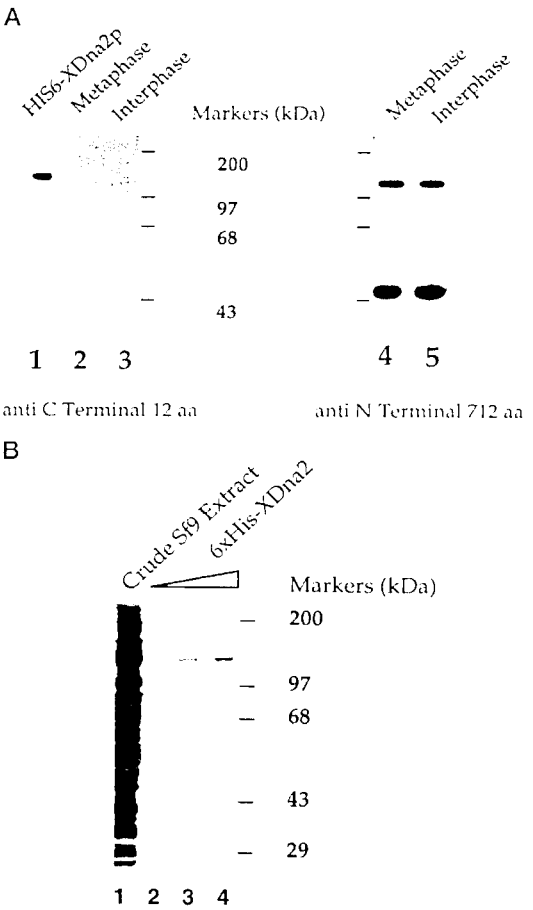

FIG. 2. **Detection of endogenous XDna2p and purification of recombinant His_6-XDna2p from insect cells.** *A*, detection of endogenous XDna2p from egg extracts by Western blotting. Proteins in metaphase and interphase egg extracts were separated by SDS-polyacrylamide gel electrophoresis. Gels were blotted and filters were probed using either affinity-purified polyclonal antibody against the C-terminal 12 amino acids of XDna2p (*lanes 1–3*) or affinity-purified polyclonal antibody against the N-terminal 712 amino acids of XDna2p (*lanes 4* and *5*). *Lane 1*, recombinant His_6-XDna2p as a positive control. *Lanes 2* and *4*, metaphase egg extracts. *Lanes 3* and *5*, interphase egg extracts. *B*, silver-stained SDS-polyacrylamide gel of purified His_6-XDna2p. *Lane 1*, crude extract; *lanes 2–4*, 10, 25, and 50 ng of purified protein, respectively.

known Dna2 functions, since the N-terminal 105 amino acids of ScDna2p can be deleted without loss of *in vivo* function, and the N-terminal 400 amino acids of ScDna2p can be deleted without affecting its known catalytic activities (3, 4, 13, 22).

Human Dna2 (HDna2p), *C. elegans* Dna2 (CDna2p) and ScDna2p are 63, 33, and 34% identical to XDna2p, respectively (Fig. 1*B*). A recent data base search revealed another Dna2 homolog (GenBank™ AC003981 or AC000106) from *Arabidopsis thaliana*, which is 37% identical to XDna2p. CDna2 is the only gene encoding a protein similar to ScDna2p in the *C. elegans* complete genome sequence, suggesting that *DNA2* is a unique gene even in metazoans, like other essential replication genes, such as those encoding the DNA polymerases. Alignment of sequences of XDna2p, HDna2p, ScDna2p, ADna2p, and

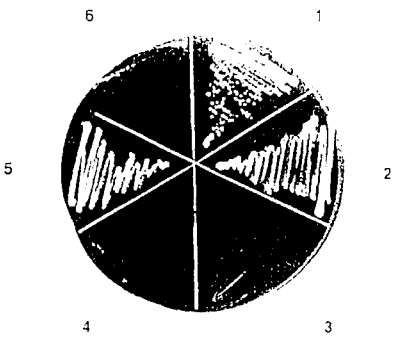

FIG. 3. **The XDna2 gene can complement *S. cerevisiae dna2-1*.** Plates carrying either wild type or *dna2-1* mutant yeast transformed with various plasmids were incubated at 37 °C. *Sectors 1*, *5*, and *6*, *dna2-1* transformed with pGALXDna2, pGALScDna2, or pSEY18-GAL, respectively; *sector 2*, wild type transformed with pGALXDna2; *sectors 3* and *4*, the same transformants as *sectors 6* and *1*, respectively, but streaked after growth on 5-fluoroorotic acid plates, which select for isolates that have lost the Ura^+ plasmids. pGALScDna2 was a construct in which the Sc*DNA2* gene was cloned into pSEY18-GAL.

CDna2p revealed many conserved residues over the entire length of the protein. In Fig. 1*C*, the five major (based on the high concentration of identical residues) conserved domains, I–V, are shown. Each domain, in turn, contains some shorter motifs that are even more similar to each other. In particular, XDna2p contains the five helicase motifs of ScDna2p (motifs 4–8, Fig. 1*A*) within domains III–V. The proline residue in motif 1 of domain I is strictly conserved, corresponds to the proline mutated in *S. cerevisiae* mutant *dna2-1*, and therefore probably identifies a critical functional motif within all Dna2 species. A number of acidic residues conserved in the N-terminal half of the protein may contribute to nuclease function (23). (Motifs 2 and 3 in Fig. 1*A* are the two conserved motifs used to design the two degenerate primers for PCR.)

Detection of Endogenous XDna2p from Egg Extracts—Polyclonal antibodies against the C-terminal 12 amino acids of XDna2p or against the N-terminal 712 amino acids were affinity-purified using agarose beads coupled to the C-terminal peptide or the recombinant N-terminal fragment, respectively. When used to probe Western blots of protein from metaphase and interphase *Xenopus* egg extracts, the C-terminal antibody detected a single protein band of 116 kDa from both metaphase and interphase egg extracts (Fig. 2*A*, *lanes 2* and *3*). We conclude that this is the XDna2 gene product, because it migrates slightly faster than the recombinant His_6-XDna2p expressed in insect cells (Fig. 2*A*, *lane 1*), which is slightly larger because of the presence of the histidine tag at the N terminus of XDna2p. The antibody against the N-terminal 712 amino acids detected two protein bands (Fig. 2*A*, *lanes 4* and *5*). The larger protein is 116 kDa and is probably intact XDna2p. The smaller one is about 43 kDa. It is not clear whether this 43-kDa protein is a proteolytic fragment of XDna2p, but it does not appear to increase in abundance as the extracts age. Furthermore, it cannot be immunoprecipitated with the affinity-purified antibody against the N-terminal 712 amino acids.

XDna2 Complements S. cerevisiae dna2-1 Mutants for Growth at the Nonpermissive Temperature—S. cerevisiae dna2-1 mutants do not grow at 37 °C. When a plasmid containing a *URA* selectable marker and the XDna2 gene, under the control of the yeast galactose-inducible promoter, *GALI,10*, was transformed into *dna2-1*, the ability of the *dna2-1* strain to grow at 37 °C was restored (Fig. 3, *sector 1*). Removal of the *URA* plasmid from the transformants by plating on medium

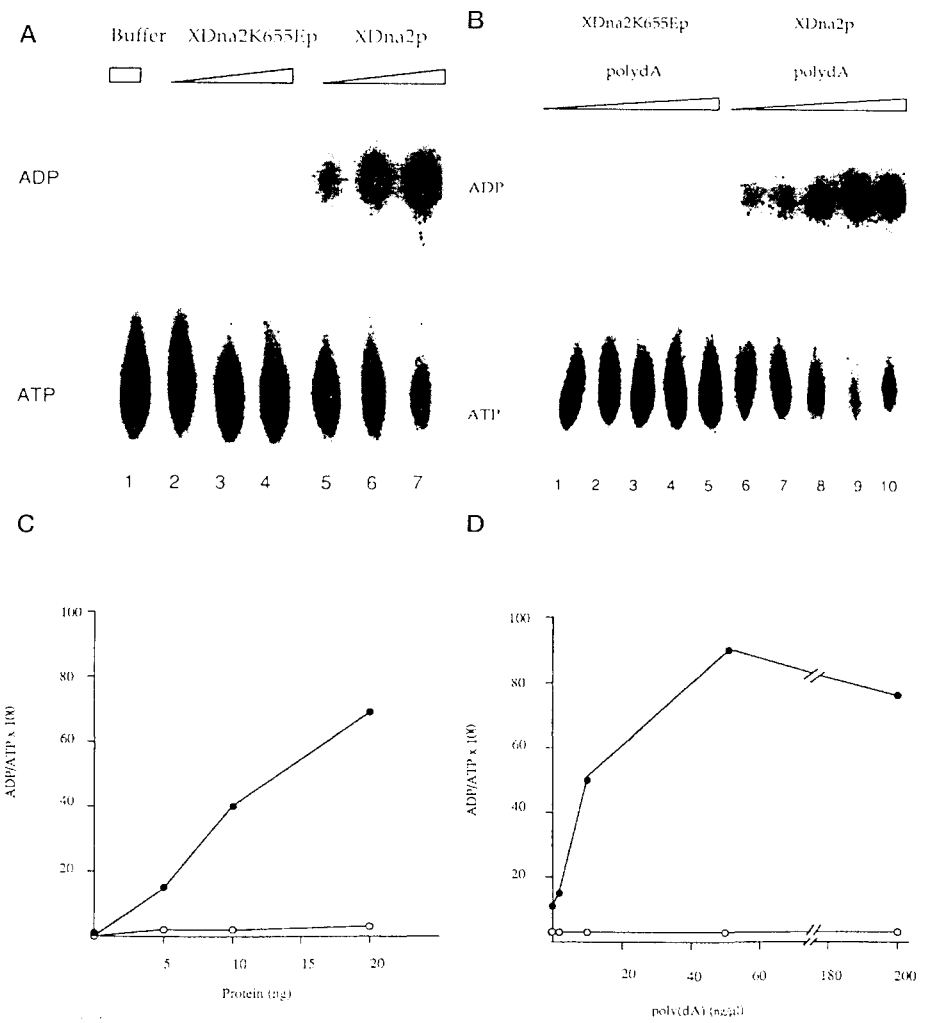

FIG. 4. **XDna2p is a DNA-dependent ATPase.** Purified His_6-XDna2p or His_6-K655Ep was incubated with [α-^{32}P]ATP for 1 h at 37 °C in a buffer described under "Experimental Procedures." The products were spotted onto a polyethyleneimine-cellulose plate that was then developed in 0.5 M LiCl, 1.0 M formic acid solution. The amount of both ATP and ADP on the plates was quantified using the PhosphorImager. *A*, ATPase activity. 0, 5, 10, and 20 ng of either purified XDna2p or mutant XDna2K655Ep was used in the ATPase assays. Poly(dA) (50 ng/μl) was present in all reactions. *B*, DNA dependence of ATPase activity. Reactions contained 0, 2, 10, 50, or 200 ng/μl poly(dA) and 20 ng of either purified XDna2p or mutant pK655E. *C*, PhosphorImager quantitation of ATPase assay shown in *A*. *Filled circles*, His_6-XDna2; *open circles*, restart His_6-XDna2K655Ep. *D*, PhosphorImager quantitation of data in *B*. *Filled circles*, His_6-XDna2p; *open circles*, His_6-XDna2K655Ep.

containing 5-fluoroorotic acid, which selects against Ura$^-$, led to loss of suppression (Fig. 3, *sector 4*), demonstrating that growth was not due to recombination between XDna2 on the plasmid and yeast chromosome copies of *dna2-1*. Vector without the XDna2 gene could not support the growth of *dna2-1* (Fig. 3, *sectors 3* and *6*). Complementation by XDna2 was as efficient as complementation by Sc*DNA2* (Fig. 3, compare *sectors 1* and *5*). Overproduction of Sc*DNA2* has been reported to cause growth defects in *S. cerevisiae* (15). However, overexpression of the XDna2 gene in a wild type yeast strain had no obvious toxic effect (Fig. 3, *sector 2*). Therefore, the XDna2p can carry out the essential function of ScDna2p, which strongly suggests that XDna2 is a functional as well as structural homolog of Sc*DNA2*.

XDna2p Is a DNA-dependent ATPase—To study its biochemical activities, XDna2p was fused to a histidine tag and expressed in insect Sf9 cells using the Bac to Bac Baculovirus system from Life Technologies, Inc. His_6-XDna2p was purified by Ni^{2+}/NTA affinity chromatography using stringent conditions (5 mM EGTA was added into the lysis buffer, and 60 mM imidazole was added into the washing buffer). The purity of the protein is evaluated in Fig. 2*B*.

Purified His_6-XDna2p had ATPase activity (Fig. 4A, *lanes 5–7*, and Fig. 4*C*) that was greatly stimulated by poly(dA) (Fig.

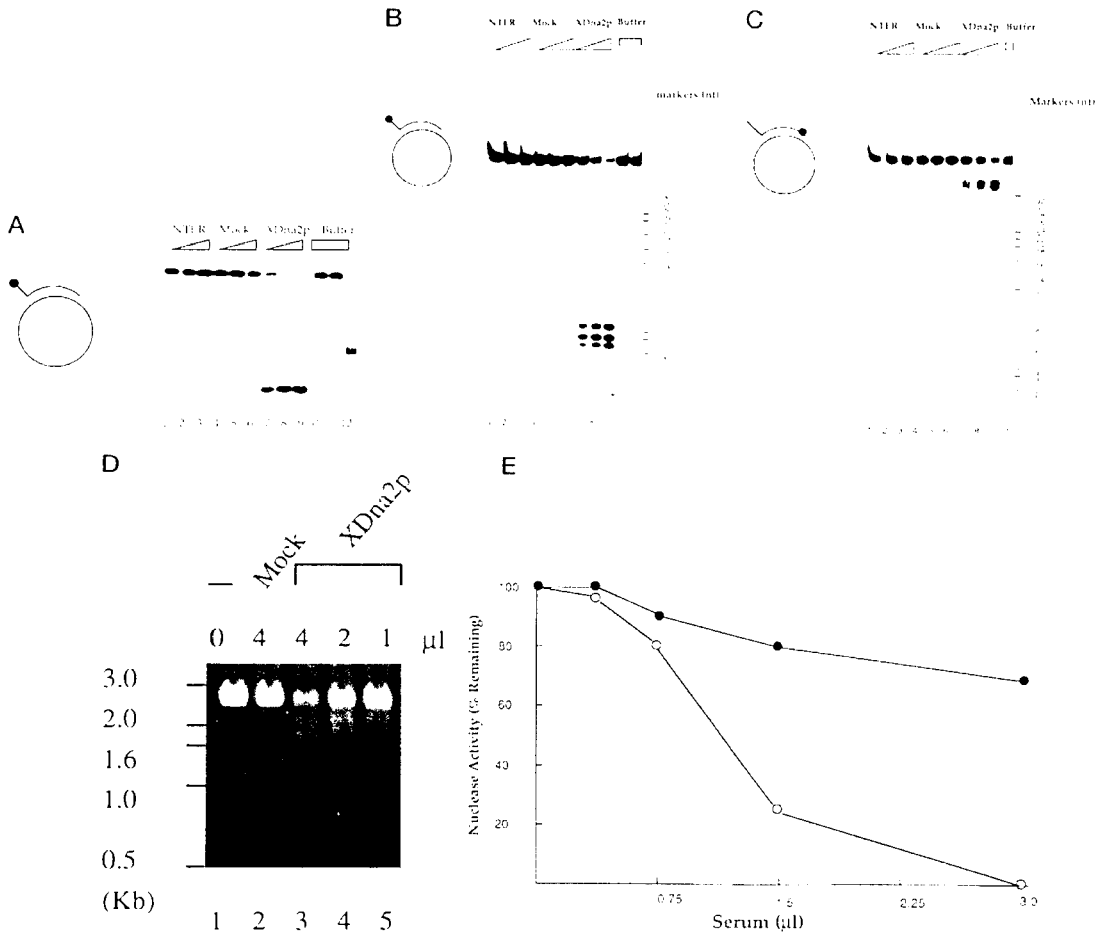

FIG. 5. **XDna2p is a single-stranded DNA endonuclease.** *A*, helicase assay reveals nuclease activity only. A 5′-end-labeled oligonucleotide annealed with M13 ssDNA (shown on the *left*) was incubated with increasing concentrations of His_6-NTERp (N-terminal 556-amino acid fragment of XDna2p) (*lanes 1–3*), Ni^{2+}/NTA column eluate from mock-infected cells (*lanes 4–6*), or His_6-XDna2p (*lanes 7–9*) for 30 min at 37 °C. The products were loaded onto an 8% polyacrylamide gel (prepared in TBE buffer) and electrophoresed. The gel was then dried and analyzed using the PhosphorImager. No protein was added to the reaction for *lanes 10* and *11*, which are the same. *Lane 12* shows the boiled substrate. *B*, the same products eletrophoresed on a 20% sequencing gel to determine the size. *C*, 3′-end-labeled oligonucleotide annealed with M13 ssDNA (*left part* of *C*; the *filled circle* indicates the radiolabel) was used as the substrate. Products were analyzed on a 20% sequencing gel. *D*, endonuclease assay with single-stranded circular DNA. Single-stranded M13 DNA (1 µg) was incubated with purified recombinant His_6-XDna2p in 20 µl of reaction buffer for 30 min at 37 °C. Products were then electrophoresed in a 0.9% agarose gel and stained with ethidium bromide. *Lane 1*, buffer only; *lane 2*, purified protein from mock-infected cells, equivalent to *lane 3*; *lane 3*, 40 ng of His_6-XDna2p; *lane 4*, 20 ng of His_6-XDna2p; *lane 5*, 10 ng of His_6-XDna2p. *E*, inhibition of nuclease by antibody. His_6-XDna2p was incubated with preserum or antiserum against the N-terminal 712 amino acids of XDna2p on ice for 10 min in nuclease assay buffer. The 3′-end-labeled substrate (see Fig. 5*C*) was added, and incubation was continued for 30 min at 37 °C. Products were boiled, loaded onto a 8% polyacrylamide gel prepared in 1× TBE buffer, and electrophoresed. The gel was dried, and quantitation was carried out using the PhosphorImager. *Filled circles*, preserum; *open circles*, XDna2p antiserum.

4*B*, *lanes 6–10*, and Fig. 4*D*). The optimum concentration of poly(dA) was 1.5 mM (Fig. 4*B*, *lane 9*). A mutant of XDna2p in which amino acid residue 655 (a lysine in the Walker A box, a putative NTP binding motif) was changed to glutamic acid, His_6-XDna2K655Ep, was purified in parallel. The analogous mutation has been shown previously to inactivate both the DNA-dependent ATPase and the helicase activity of ScDna2p (2). The His_6-XDna2K655E mutant preparation had a barely detectable background of ATPase activity (Fig. 4*A*, *lanes 2–4*, and Fig. 4*C*), and this activity was not stimulated by DNA (Fig. 4*B*, *lanes 1–5*, and Fig. 4*D*). Thus, we conclude that the ATPase observed in the His_6-XDna2p preparation is due to XDna2p. The of XDna2p ATPase was estimated to be 0.01 pmol/ng/min, which is about 500-fold lower than the specific activity of recombinant ScDna2p purified from insect cells.[5]

XDna2p Is an Endonuclease That Can Degrade Single-stranded DNA—Highly purified ScDna2 overexpressed in *S. cerevisiae* was shown to have a 3′ to 5′ DNA helicase activity and copurified with a nuclease activity (2). When the purified recombinant XDna2p expressed in insect cells was incubated with a substrate consisting of M13 DNA annealed to a 44-mer

[5] W. Choe and J. L. Campbell, unpublished results.

oligonucleotide with a 14-nucleotide noncomplementary 5′ tail (Fig. 5*A*, *left side*), no helicase activity was detected. However, the oligonucleotide, which was labeled at the 5′ terminus, was converted to a rapidly migrating species of less than 10 nucleotides in length (Fig. 5*A*, *lanes 7–9*), indicating that a nuclease activity was associated with XDna2p. This activity was not present when extracts of cells infected with baculoviruses lacking an insert were carried through the same affinity purification procedure (Fig. 5*A*, *lanes 4–6*, *Mock*) and thus appears to be intrinsic to XDna2p. It seemed reasonable that the nuclease domain of XDna2p might be located in the N-terminal half, since the C-terminal portion of ScDna2p was required for helicase activity. To test this idea, the N-terminal 556-amino acid fragment (NTER) of His_6-XDna2p was also expressed and purified. However, no nuclease activity was detected (Fig. 5*A*, *lanes 1–3*), and therefore the location of the nuclease catalytic site remains unknown. From Fig. 5*A*, it appeared that recombinant XDna2 expressed in insect cells did not have helicase activity. Since it is possible that failure to detect helicase activity is due to the strong nuclease activity that destroys the 5′-labeled substrate, 3′-labeled substrate was also used to test the helicase activity of recombinant XDna2. However, no helicase activity was detected.

To characterize the nuclease specificity, the size of the products was determined by electrophoresis through a 20% sequencing gel (Fig. 5*B*). The major products were oligomers of 5, 6, and 7 nucleotides (Fig. 5*B*, *lanes 7*, *8*, and *9*), suggesting that the nuclease might cut within the single-stranded tail of the labeled oligonucleotide. No products in this size range are produced when purified NTER or protein from mock-infected cells were used. Identical products were observed when the labeled 44-mer oligonucleotide was incubated with XDna2p in the absence of M13 (data not shown). Thus, the enzyme is a single-strand-specific nuclease rather than a structure-specific nuclease requiring a single-stranded region adjacent to a duplex region. A small amount of radioactivity migrated with the salt front (Fig. 5*B*, *lanes 1–6*), suggesting the presence of a minor contaminating nuclease or phosphatase activity in the purified XDna2p sample. Chromatography on heparin Sepharose followed by a Mono Q column did not eliminate this background. Next, the products generated with the same 44-mer/M13 substrate, but labeled at the fully duplex 3′-terminus using Klenow enzyme and [α-^{32}P]dTTP, were analyzed. The size of the products was greater than 32 nucleotides, the length of the fully annealed region of the oligonucleotide (*lanes 7–9*, Fig. 5*C*). The products from XDna2p digestion appeared as one band (the bands of >32 nt in *lanes 7-9*, Fig. 5*C*), because the resolving power of the gel was not high enough to separate the larger species. No other product was detected with this substrate. Thus, XDna2p specifically cleaved the 5′ single-stranded tail of the oligonucleotide, and XDna2p is a single-stranded DNA endonuclease, consistent with the data for ScDna2p (4). In Fig. 5*D*, digestion of circular, single-stranded M13 DNA by His_6-XDna2p confirms that it is an endonuclease and does not require ends.

To ensure that the nuclease activity was due to XDna2p, the 3′-labeled substrate (the same as for Fig. 5*C*) was incubated with purified XDna2p in the presence of different concentrations of XDna2p antiserum or preantiserum (Fig. 5*E*). The products were then electrophoresed and quantified using PhosphorImager analysis. When 3 μl of antiserum was added, the nuclease activity was completely inhibited, but most (70%) of the nuclease activity was still there in the presence of 3 μl of preantiserum, providing further evidence that XDna2p was associated with a nuclease activity. The activity that produced the material migrating at the front of the gel in Fig. 5*B* was not inhibited when the 5′-end-labeled substrate was used (not shown), further suggesting that this "activity" is not due to the action of XDna2p on the substrate.

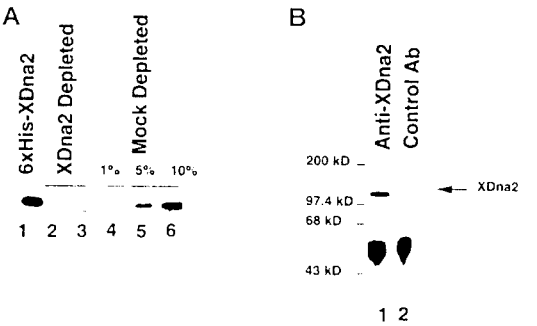

FIG. 6. **Immunodepletion of XDna2 from interphase extracts.** 20 μl of protein A beads were incubated with 20 μg of either affinity-purified anti-XDna2 antibody or a control antibody for 1 h at room temperature and then were washed with 1× HBS buffer. 20 μl of interphase extract was incubated with 10 μl of beads from above for 45 min at 4 °C. The supernatant was recovered and incubated with a fresh aliquot of beads (10 μl) for 45 min at 4 °C. The supernatant and the pellet were then analyzed by Western blotting using antiserum against XDna2. *A*, *lane 1*, recombinant His_6-XDna2 expressed in insect cells, which migrates slightly slower than the endogenous XDna2 shown in *lanes 4–6* because of the tag; *lanes 2* and *3*, XDna2-depleted extract (2 μl of supernatant); *lanes 4–6*, 1, 5, and 10% of 2 μl of mock-depleted extracts were loaded. *B*, *lane 1*, the pellet from immunodepletion using the antibody against XDna2; *lane 2*, the pellet from immunodepletion using the control antibody.

Recombinant ScDna2p purified from insect cells displays a weak ATP-dependent nuclease activity that degrades double-stranded DNA from 3′-ends (4) and that is also found in ScDna2p prepared from yeast, unless thio-substituted oligonucleotides are used in the assays (3).[2] ATP was present in the nuclease assays described above, but no 3′ nuclease was detected (see Fig. 5*C*). Furthermore, the results of the nuclease assays were the same when ATP was absent (data not shown). Thus, XDna2p seems to lack an activity specific for 3′ duplex ends. Since the ATPase activity of XDna2p was much lower than that of ScDna2p, it is difficult to rule out the possibility that the ATP-dependent nuclease, which is a very minor activity compared with the single-stranded endonuclease, was inactivated during expression and purification of recombinant XDna2p.

Depletion of XDna2 from Interphase Egg Extracts Strongly Inhibits Chromosomal DNA Replication, but Not M13 Single-stranded DNA Repair Synthesis—Immunodepletion of interphase egg extracts that faithfully replicate sperm chromatin was carried out to test whether XDna2 is involved in DNA replication in *Xenopus*. Extracts were prepared for DNA replication as described under "Experimental Procedures." Extracts were then incubated with affinity-purified XDna2 antibody to remove XDna2 present in the extracts. From Western blot analysis of the immunoprecipitate and the supernatant after depletion, we conclude that XDna2 antibody effectively immunoprecipitates XDna2 but that mock depletion does not (Fig. 6). (The *upper band* in *lane 1* of Fig. 6*B* is XDna2, and the *lower band* is IgG.) More than 95% of XDna2 is removed from the extract by this antibody compared with extract treated with control antibody (Fig. 6*A*, compare *lanes 2* and *3* with *lanes 4–6*). Depleted and mock-depleted extracts were then assayed for replication of sperm chromatin. Demembranated sperm and [α-^{32}P]dCTP were added into the XDna2-depleted or mock-depleted interphase egg extracts, and then the mixtures were

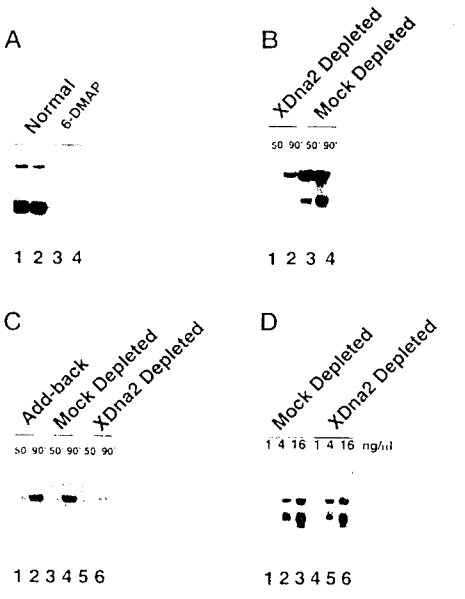

FIG. 7. **Chromosomal DNA replication is inhibited in XDna2-depleted extracts.** Extracts were prepared as described under "Experimental Procedures." *A*, 6-DMAP can inhibit DNA replication in extracts. 10 μl of normal interphase extract (*lanes 1* and *2*) or 6-DMAP-treated extract (*lanes 3* and *4*) was incubated with 0.5 μl of [α-^{32}P]dATP and sperm nuclei (1000 nuclei/μl final concentration) for 1 h at room temperature. Subsequently, the reaction was stopped by the addition of 10 μl of 80 mM Tris·HCl (pH 8.0), 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, and 0.2% bromphenol blue. Proteinase K was added to digest the protein in the mixture. The DNA samples were loaded onto a 1% agarose gel and electrophoresed. The gel was dried and analyzed by PhosphorImager analysis. *B*, the same DNA replication assay as described in *A* was carried out except that XDna2-depleted extract (*lanes 1* and *2*) or mock-depleted extract (*lanes 3* and *4*) was used in the assay, and the replication reaction was carried out for 50 min (*lanes 1* and *3*) or 90 min (*lanes 2* and *4*). The immunodepletion is described under "Experimental Procedures." *C*, the addition of 5% mock-depleted extract into the depleted extract can restore replication efficiency. *Lanes 1* and *2*, depleted plus 5% mock-depleted extract; *lanes 3* and *4*, mock-depleted extract; *lanes 5* and *6*, XDna2-depleted extract. *D*, Single-stranded M13 DNA instead of sperm nuclei was used to test the efficiency of DNA synthesis in the XDna2-depleted (*lanes 4–6*) or mock-depleted extract (*lanes 1–3*). The replication assay procedure is the same as described in *A*. The final concentration of single-stranded M13 DNA in the reaction is 1 ng/μl for *lanes 1* and *4*, 4 ng/μl for *lanes 2* and *5*, and 16 ng/μl for *lanes 3* and *6*.

incubated at room temperature for 50 and 90 min, respectively, before the termination of reactions. To verify that the synthesis observed in the *in vitro* system represented regulated DNA replication, we included reactions containing the cyclin-dependent kinase inhibitor 6-DMAP, which specifically inhibits chromosomal DNA replication in the egg extracts. As shown in Fig. 7*A*, synthesis in the extracts is efficient and occurs with the expected timing. Furthermore, synthesis is entirely inhibited by 6-DMAP. Synthesis was then measured in the depleted and mock-depleted extracts. Chromosomal DNA replication efficiency of the XDna2 depleted egg extracts was decreased by at least 90% (*lanes 1* and *2*) compared with the mock depleted egg extracts (*lanes 3* and *4*), suggesting that XDna2 is required for chromosomal DNA replication. The formation of nuclei was normal as monitored by staining with Hoechst and fluorescence microscopy (data not shown).

An important question is whether the inhibition observed is due to the specific removal of XDna2. Restoration of synthesis by the addition of purified enzyme would argue for such specificity. The addition of recombinant XDna2 expressed in insect cells, however, failed to restore the DNA replication efficiency of the depleted extracts (data not shown). Nevertheless, we were able to restore synthesis completely by adding back a small sample of mock-depleted extract (5% final concentration) into the depleted extract (Fig. 7*C*, compare *lanes 1* and *2* with *lanes 3–6*). Failure of the purified recombinant protein to complement could be explained if additional essential proteins are coimmunodepleted together with XDna2. It is known that ScDna2 interacts with both ScFEN1 and ScCtf4 proteins, for instance. Since the recombinant XDna2 does not have DNA helicase activity, it is also possible that the recombinant XDna2 is not correctly folded or lacks appropriate post-translational modifications. When antiserum against XDna2, rather than affinity-purified antibody, was used for immunodepletion of the interphase egg extracts, chromosomal DNA replication was also inhibited by at least 90% compared with mock-depleted egg extracts prepared using the corresponding preimmune serum (data not shown). That two different depletion protocols yielded similar results also strengthens the conclusion that the defect in DNA replication in the XDna2-depleted egg extracts is due to the specific removal of XDna2.

Another way to test the specificity of the defect in the depleted extract is to show that nonreplicative DNA synthesis is normal. The synthesis of the complementary strand of M13 single-stranded DNA has been used as such a control (21, 24). In XDna2-depleted extract, synthesis of the M13 complementary strand is completely normal (Fig. 7*D*). Although DNA replication of sperm chromatin is almost completely inhibited after depletion of XDna2, the extracts are still capable of extensive priming and synthesis of DNA on templates that do not require assembly of a replication fork.

Genetic interactions between ScDna2 and ScRad27 have led to the proposal that Dna2 may play a role in Okazaki fragment processing. If XDna2 is required for processing Okazaki fragments, then one would expect the accumulation of short DNA fragments during DNA synthesis in extracts lacking XDna2, as is observed when extracts are treated with the DNA polymerase inhibitor aphidicolin (25, 26). If XDna2 is required for an earlier stage of replication, then one would expect the residual DNA synthesized to be full-length, as is observed when extracts are depleted of origin recognition complex (27). Denaturing agarose gel electrophoresis was used to measure the size of the DNA synthesized in the mock-depleted egg extracts (Fig. 8, *lanes 1* and *2*) and in the XDna2-depleted extracts (Fig. 8, *lanes 3* and *4*). The small amount of DNA synthesized was all full-length, and there was no accumulation of Okazaki fragments in the XDna2-depleted egg extracts. This suggests that the frequency of initiation is reduced in the depleted extracts but that the initiations that did occur could proceed to completion.

DISCUSSION

Using a degenerate PCR probe, we cloned XDna2 from a *Xenopus* oocyte cDNA library. XDna2 encodes a protein of 1053 amino acids. We believe that XDna2 is a true homolog of *ScDNA2*, based on the following observations: 1) the deduced amino acid sequence of XDna2p was 32% identical to ScDna2p, and conserved motifs are located throughout the N-terminal domain in addition to the C-terminal helicase domain; 2) the XDna2 gene could efficiently complement *Scdna2-1* mutants for growth at the nonpermissive temperature; 3) the recombinant XDna2p has both the DNA-dependent ATPase and single-stranded DNA endonuclease activities associated with ScDna2p; and 4) depletion of XDna2 from egg extracts inhibits replication of sperm chromatin. The presence of homologous

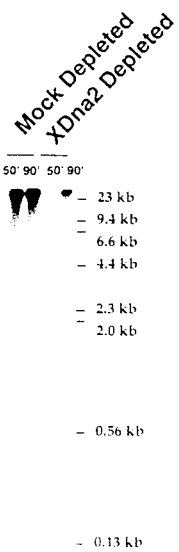

FIG. 8. **Analysis of the DNA replication products from the XDna2-depleted or mock-depleted extracts after denaturation on an alkaline agarose gel (1%).** The preparation of DNA replication products is the same as described in the legend to Fig. 7*B*. *Lanes 1* and *2*, DNA products synthesized in mock-depleted extract; *lanes 3* and *4*, DNA products synthesized in the XDna2-depleted extracts. *Lanes 1* and *3*, 50 min; *lanes 2* and *4*, 90 min.

sequences from human, *C. elegans*, and *Arabidopsis* in the data base indicate that ScDna2p is evolutionarily conserved and suggest that the Dna2 protein must play a very important role in eukaryotic DNA metabolism. With the *C. elegans* sequence virtually complete, it is also interesting to point out that CDna2p is not similar to any other protein in the worm genome and thus serves a unique function.

While the similarities are striking, there are also differences between XDna2p and ScDna2p. Most obvious is the absence of helicase activity on forked substrates. This may not be a real difference, however, since recombinant ScDna2p purified from insect cells also lacked helicase activity (4). Only the ScDna2p actually purified from yeast was active as a helicase (2). It may be that the recombinant XDna2p was not correctly folded, assembled into oligomeric form, or posttranslationally modified. It is also possible that the binding of another unknown factor is important for its helicase activity (2–4). A second difference is that the weak ATP-dependent nuclease activity of recombinant ScDna2p was not detected in recombinant XDna2p. Since the specific ATPase activity of XDna2p was only 0.01 pmol/min/ng, 500-fold less than ScDna2p, the activity may be very difficult to detect. A third difference is that ScDna2p (1522 amino acids) has 468 amino acids at its N terminus that are not found at all in the XDna2 open reading frame. Much of this region in ScDna2p, 405 amino acids, can be deleted without reducing any of the biochemical activities of wild-type ScDna2p (4), and it is possible that more amino acids can be deleted from the N terminus without abolishing activities of ScDna2p. It is not known if the truncated form can support growth, however. This domain is also absent from the *Arabidopsis* and *C. elegans* cDNAs. The function of the unique N-terminal domain of ScDna2p is currently unknown.

Copurification of ScDna2p with a nuclease activity led to the proposal that Dna2p itself might be a nuclease as well as a helicase (2–4). While it could be shown that the structure-specific nuclease Fen1p copurified with the ScDna2p from yeast, the single-stranded nuclease activity remained in a strain carrying a *rad27Δ*, indicating that at least one additional nuclease was present (13). It now appears that the second activity is intrinsic to Dna2p, since it is present in highly purified recombinant ScDna2p from insect cells. Also, like the yeast enzyme, XDna2p was shown here to be a single-stranded DNA-specific endonuclease that yielded short oligonucleotides as products rather than a structure-specific nuclease. Several known helicases (yeast SGS1, the Werner syndrome helicase, and the bacterial RecB,C,D enzyme) are associated with nuclease activities, although none of the known helicase/nuclease proteins has been shown, like Dna2p, to be involved in DNA replication. Like ScDna2, XDna2 falls in the RecB family of helicase nucleases based on primary structure comparison (28).

ScDna2p is required for chromosomal DNA replication, although the exact way in which it contributes has proved elusive. Since ScDna2p and XDna2p are single-stranded DNA endonucleases, and ScDna2p and ScFen1p could reciprocally suppress the temperature-sensitive growth defect of their respective mutants (13), it is possible that Dna2 proteins play a role in Okazaki fragment processing like the nuclease Fen1. Most recently, it was shown that both overexpression of ScDna2 and deletion of ScFen1 yielded single-stranded DNA regions on telomeric DNA. Since the accumulated single-stranded DNA was the templating strand for lagging-strand synthesis, it was suggested that ScDna2 and ScFen1 collaborate in the processing of Okazaki fragments (14). A role for Dna2 in a late stage of DNA replication is further supported by the fact that *dna2* mutant strains arrest with an apparent 2C DNA content despite the fact that *dna2* mutants fail to synthesize high molecular weight DNA at the restrictive temperature (3, 5). ScDNA2 genetically interacts with Pol1p and Ctf4p, which encode a DNA polymerase α subunit and an associated protein (15–17). ScDna2p purified from *S. cerevisiae* contains Ctf4p.[2] *dna2-1* and *mcm10* are synthetically lethal in yeast.[3] Mutation of *mcm10* suppresses mutations in *mcm2–7* genes and is thought to be involved in initiation (18).[4] Therefore, it seems also possible that ScDna2p may be somehow involved in initiation of DNA replication. The almost complete inhibition of chromosomal DNA replication in XDna2-depleted interphase egg extracts reported here and the large size of the residually synthesized DNA also hint that Dna2 may play a role in a very early step in DNA replication. The 2C DNA content in the arrested yeast *dna2* mutant cells may be due to leaky mutations. The recently described soluble nucleoplasmic extract system, which is capable of DNA replication on plasmid templates as well as sperm chromatin, will probably give us a clear answer about whether Dna2 is involved in the initiation, elongation, or both (29). It will be possible using plasmid templates to better analyze the stage of DNA replication that is blocked. Since *dna2* mutants of *S. cerevisiae* have elongated telomeres (15), Dna2p may also be involved in telomere replication.[5,6] Alternatively, this may reflect interaction of the lagging-strand complex containing an altered Dna2p with telomerase.

The number of proteins with helicase activity implicated in chromosomal replication is gradually increasing. It has been proposed that an MCM protein complex is the helicase for unwinding the origins for DNA replication in eukaryotic cells

[6] L. Hoopes, W. Choe, and J. L. Campbell, unpublished results.

(30). Another helicase, FFA-1 which is the *Xenopus* homolog of Werner syndrome protein, was shown to be involved in forming replication foci (31). FFA-1 is likely a nuclease too since the human homolog was proved to be a nuclease (32). It will be very interesting to elucidate the exact roles of Dna2p, the MCMs, and FFA-1 in DNA replication using a combination of yeast genetics and the *in vitro Xenopus* DNA replication system.

Yeast *dna2* mutants, in addition to being defective in DNA replication, are sensitive to DNA-damaging agents, especially x-rays and methylmethane sulfonate. Therefore, Dna2 may have multiple functions in DNA metabolism, like the FEN1, Mre11, and Exo1 nucleases (12, 33–43). Mutations in DNA helicases and nucleases can cause cancer-prone hereditary diseases including Bloom syndrome, Werner syndrome, and xeroderma pigmentosum (35, 41, 44–46). A more detailed study of the Dna2 protein family may have clinical in addition to theoretical significance.

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Martin E. Budd, **Won-chae Choe**, and Judith L. Campbell

From the Braun Laboratory, California Institute of Technology, Pasadena, California 91125

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Martin E. Budd, Won-chae Choe, and Judith L. Campbell‡

From the Braun Laboratory, California Institute of Technology, Pasadena, California 91125

***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′ nonhybridized 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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‡ To whom correspondence should be addressed: Braun Laboratory 147-75, California Institute of Technology, Pasadena, CA 91125. Tel.: 626-395-6053; Fax: 626-405-9452; E-mail: jcampbel@cco.caltech.edu.

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

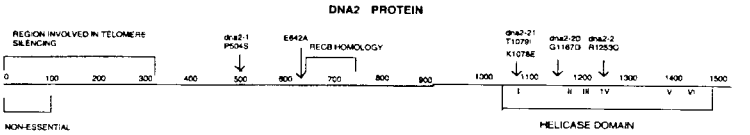

FIG. 1. **Schematic diagram of the yeast *DNA2* gene.**

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-*Sho*I 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 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).

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

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+}.

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-

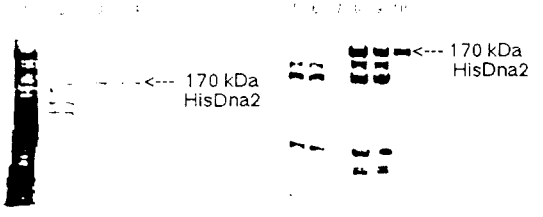

FIG. 2. **Purification of Dna2 from baculovirus-infected insect cells.** 6xHis-Dna2 was purified from insect cells infected by recombinant 6xHis-Dna2 baculovirus as described under "Experimental Procedures" and in Table I. *Lanes 1–4*, silver-stained gel. *Lane 1*, cell extracts; *lane 2*, Ni^{2+}/NTA 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.

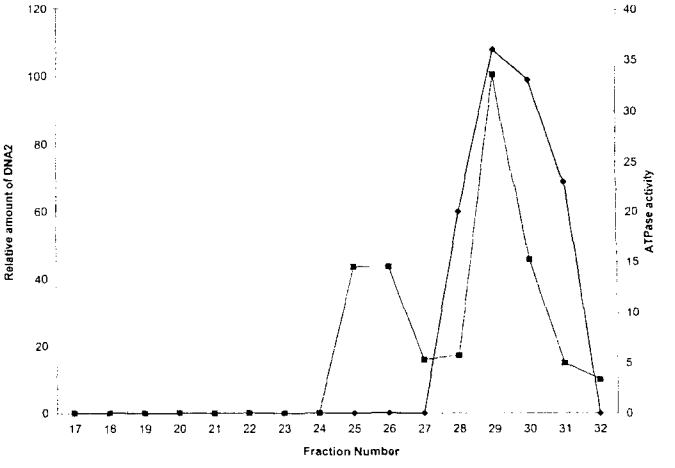

FIG. 3. **Size exclusion analysis of Dna2.** Partially purified Dna2 (0.5 mg, Ni^{2+}/NTA-agarose fraction) was loaded onto an FPLC Superose 6 HR 10/30 column. Fractions (0.5 ml) were collected and analyzed by Western blotting using anti-Dna2 polyclonal antibody. The relative amount of Dna2 in each fraction 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.

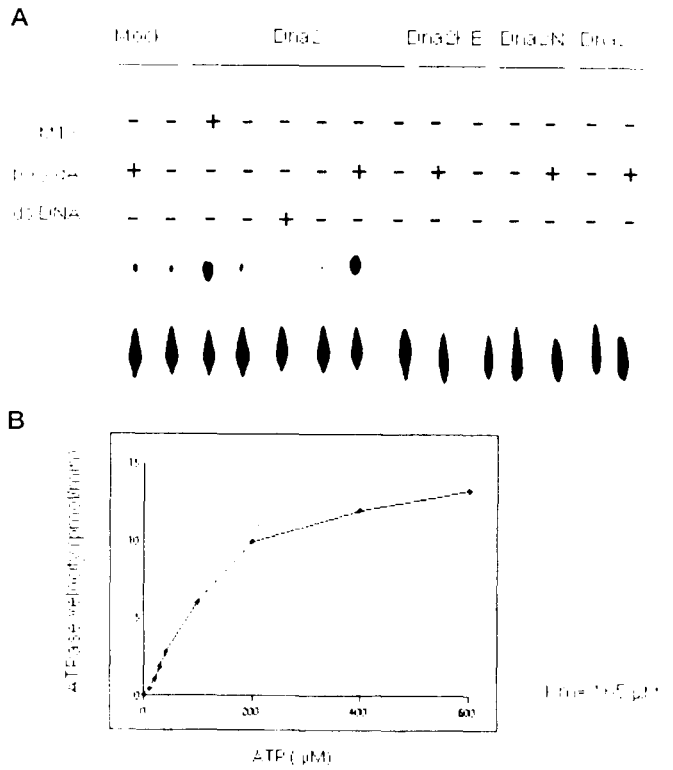

FIG. 4. **ATPase activity of purified Dna2 and mutant proteins expressed in baculovirus-infected insect cells.** *A*, DNA requirement. 6xHis-Dna2 (2 ng) was incubated with various DNA cofactors. *M13*, double-stranded DNA (linearized pRS304) and poly(dA) were each present at 2 μg. ATPase was assayed 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 *rad27Δ* 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*

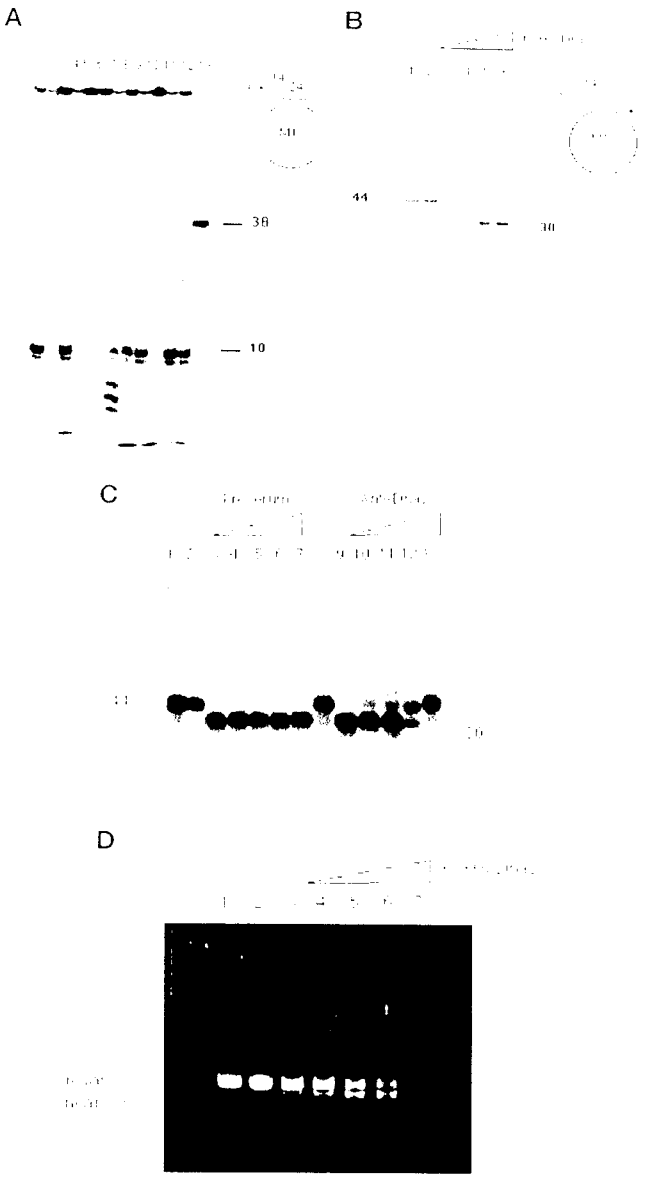

FIG. 5. **Products of endonuclease activity of wild type and mutant Dna2 proteins expressed in baculovirus-infected cells.** Oligonucleotide was annealed to M13mp18 and reactions carried out as described under "Experimental Procedures." Products were analyzed on a neutral gel. *A*, substrate labeled 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

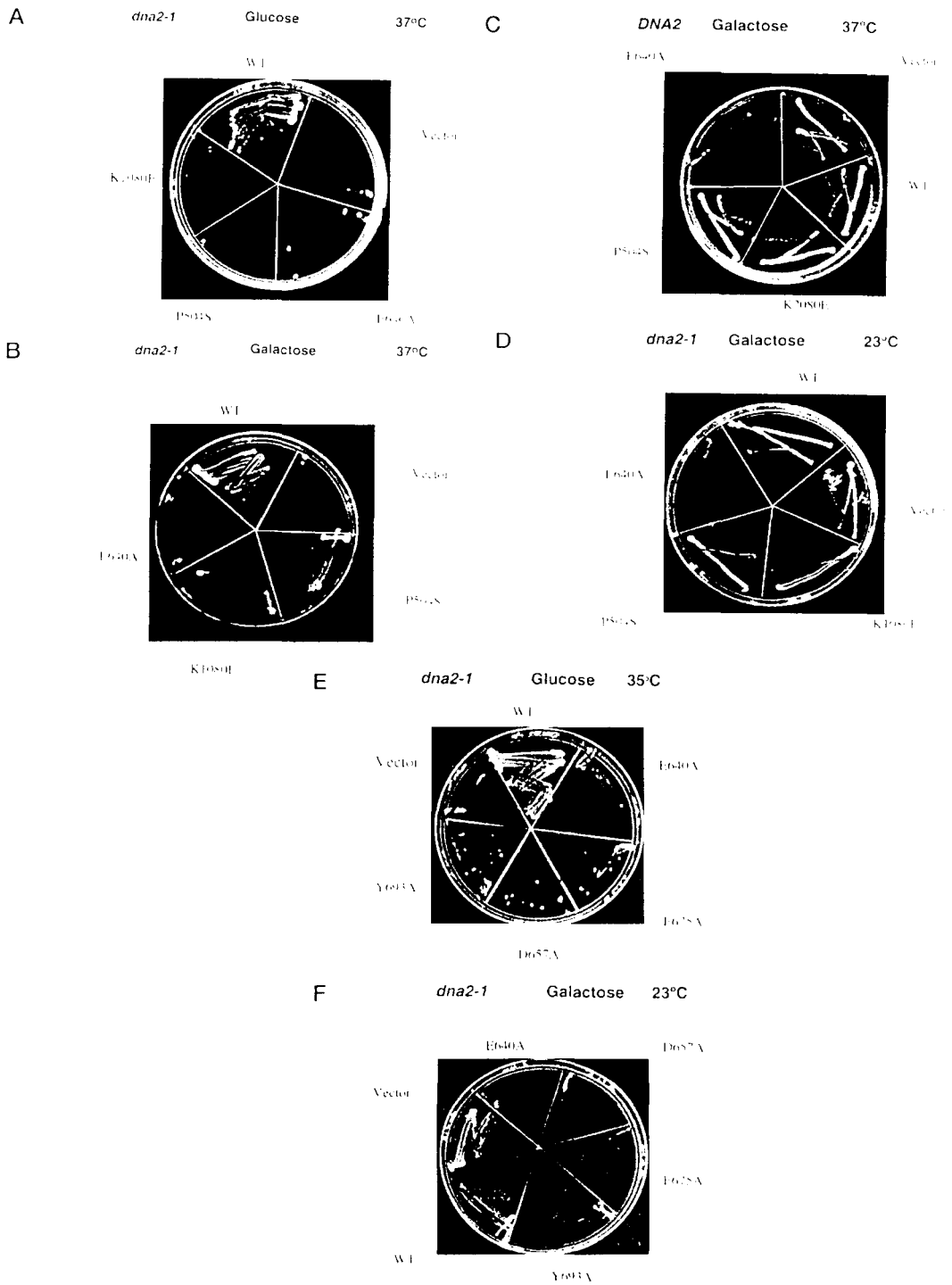

FIG. 6. **Effect of *dna2* mutations *in vivo*.** Growth and x-ray sensitivity of yeast *dna2–1* and *DNA2* strains transformed with plasmids pGAL:18, pGAL:Dna2, pGAL:Dna2:E640AA, pGAL:Dna2:P504S, and pGAL:Dna2:K1080E was measured on either glucose- or galactose-containing plates.

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, *sgs1*Δ. However, *sgs1*Δ 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 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.

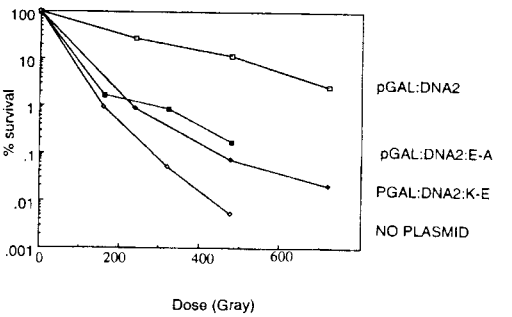

FIG. 7. **Ability of various *dna2* mutant genes to rescue x-ray sensitivity of yeast *dna2-2* strains.** *dna2-2* cells transformed with the plasmids indicated on the *right* were harvested in log phase, irradiated, serially diluted, and plated on uracil-deficient glucose-containing plates, as described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

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.

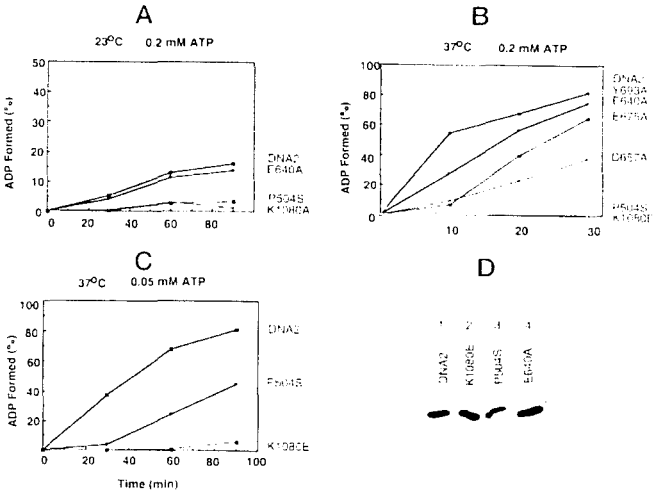

FIG. 8. **DNA-stimulated ATPase activity of wild type and mutant Dna2 proteins purified from yeast.** *A* and *B*, ATPase assays of Ni^{2+}/NTA-agarose-purified proteins. Assays contained ATP at 0.2 mM and 1 μg of Ni^{2+}/NTA-agarose-purified protein. The temperature of incubation is indicated. *C* and *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.

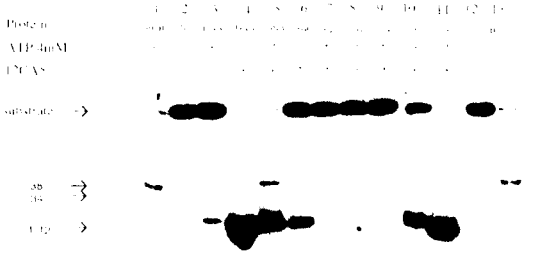

FIG. 9. **Comparison of DNA helicase activities of wild type and mutant Dna2 proteins purified from yeast.** *A*, 5 μg of Ni^{2+}/NTA-agarose-purified protein was immunoprecipitated with 5 μg of 12CA5 anti-HA antibody, followed by addition of 10 μl of 10% protein A beads. Beads were washed and assayed 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

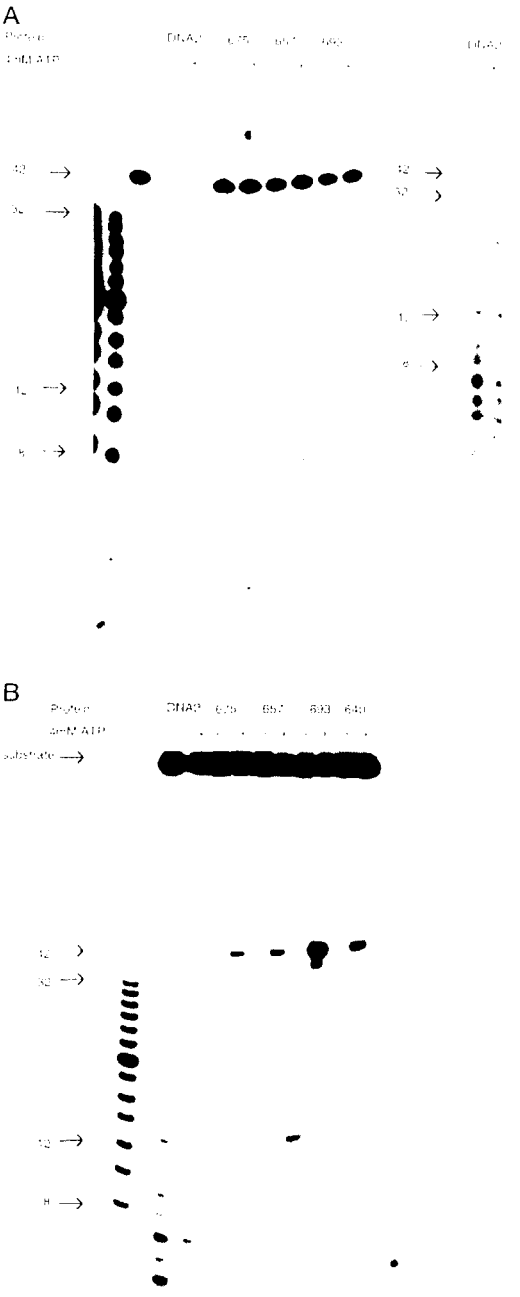

FIG. 10. **Nuclease and helicase assays of N-terminal, RecB-homologous mutants.** *A*, nuclease assays. Nuclease assays were carried out in reaction mixtures as described under "Experimental Procedures" using mutant protein purified by Ni^{2+}/NTA and 12CA5 affinity steps. The substrate was hpr7-labeled 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[c]	+/–	+/–	+/–

[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, *rad27Δexo1* mutants and *rad27mre11* mutants are, like *rad27Δdna2* mutants, synthetically lethal. Exo1 is a 5′ to 3′ double-stranded exonuclease that interacts with Msh2 in two hybrid assays (34, 35). Mre11 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. *rad27Δ rad52Δ* 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 srs2Δ* 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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Chapter 3: New Biological *In Vivo* Functions of Dna2p in *Saccharomyces cerevisiae*

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Dynamic localization of an Okazaki fragment processing protein suggests a novel role in telomere replication

Wonchae Choe, Martin Budd, Osamu Imamura, Laura Hoopes[1] and Judith L. Campbell*

Braun Laboratories, 147-75

Pasadena, CA, 91125

Telephone: 626/395-6053 Fax: 626/405-9452

E-mail: jcampbel@cco.caltech.edu

[1]Department of Biology and Molecular Biology Program

Pomona College, Seaver South Laboratory

Claremont, CA 91711

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Corresponding Author: Judith L. Campbell

ABSTRACT

We have found that the Dna2 helicase-nuclease, thought to be involved in maturation of Okazaki fragments, is a component of telomeric chromatin. We demonstrate a dynamic localization of Dna2p to telomeres that suggests a dual role for Dna2p, one in telomere replication and another, unknown function, perhaps in telomere capping. Both ChIP (chromatin immunoprecipitation) and immunofluorescence show that Dna2p associates with telomeres but not bulk chromosomal DNA in G1 phase, when there is no telomere replication and the telomere is transcriptionally silenced. In S phase, there is a dramatic redistribution of Dna2p from telomeres to sites throughout the replicating chromosomes. Dna2p is again localized to telomeres in late S, where it remains through G2 and until the next S phase. Telomeric localization of Dna2p required Sir3p, since the amount of Dna2p found at telomeres by two different assays, one hybrid and ChIP, is severely reduced in strains lacking Sir3p. The Dna2p is also distributed throughout the nucleus in cells growing in the presence of DSB-inducing agents such as bleomycin. Finally, we show that Dna2p is functionally required for telomerase-dependent de novo telomere synthesis and also participates in telomere lengthening in mutants lacking telomerase.

INTRODUCTION

S. cerevisiae Dna2p is a highly conserved helicase/nuclease essential for DNA replication (3, 10, 12-14, 30, 35, 38). It is also essential for DNA replication in *Schizosaccharomyces pombe* and in *Xenopus laevis* (34, 38). Mounting evidence indicates that Dna2p participates in the processing of Okazaki fragments, either compensating for or cooperating with the *RAD27*-encoded FEN-1 nuclease (4, 10, 12, 25, 33). Unlike most DNA replication mutants, *dna2* mutants are also defective in repair of DSBs (double-strand breaks) by the postreplication repair pathway (11, 25). *dna2* mutants require *rad9* for cell cycle arrest at the restrictive temperature (24, 25). However, the double mutants have greater viability than *dna2* mutants at semi-permissive temperatures. In this work, we describe an unprecedented type of interaction of this or any other DNA replication protein with telomeres.

Telomeres are specialized structures at the ends of chromosomes, important both for facilitating complete DNA replication and for stabilizing the ends by preventing end-to-end fusions. Yeast telomeres contain about 300 bp of heterogeneous $C_{1-3}A/TG_{1-3}$ repeats at the extreme termini. Subtelomeric repeats, called Y', are found at some but not all of the yeast telomeres, and a second set of repeats, X, are found at all telomeres (51 for review). The tandem array of $C_{1-3}A/TG_{1-3}$ repeats binds a set of specific proteins that nucleate a higher order chromatin structure that leads to silencing of genes up to several kilobase pairs internal to the terminal repeats. Such silencing is called telomere position effect (TPE), and requires *RAP1*, *SIR2*, *SIR3*, and *SIR4* genes (53). Many of the proteins are also components of the telomere capping complex that protects against fusions (6).

The majority of the chromosome terminus replicates late in S phase due to late activation of ARSs within 40 kb from the telomere (18, 22, 60). The replisome emanating from this region replicates the subtelomeric repeats and some of the $C_{1-3}A/TG_{1-3}$ repeat sequences. Recent evidence obtained from in vitro replication of a linear SV40 chromosome suggests that the eukaryotic replisome can completely copy the leading strand ($C_{1-3}A$ template strand in yeast), but that the lagging strand terminates gradually within ~50 bp from the 3' end, producing single-stranded ends (47). This is a reflection of the so-called end-replication problem, the inability to repair the gaps left by removal of the last primer or to prime the terminal Okazaki fragment on the lagging strand. Perhaps related to the observations in the SV40 system in vitro, transient single-stranded TG_{1-3} tails, 30 to 150 nucleotides long, arise late in S phase at yeast telomeres (61). Their appearance requires passage of the replication fork (20) and may involve nucleolytic processing, in addition to failure to complete the lagging strand, since both leading and lagging strand telomeres have TG_{1-3} tails (19, 61).

The end replication problem is in part solved because TG_{1-3} tails are extended by a specialized DNA polymerase, telomerase. Without telomerase, telomeres gradually shorten. The products of the yeast *TLC1*, *EST1*, *EST2* (the catalytic subunit of telomerase), *EST3*, and *CDC13 (a.k.a. EST4)* genes are required for TG_{1-3} tail extension (46 for review). TG_{1-3} tails disappear at G2/M, presumably by $C_{1-3}A$ strand fill-in, but the source of the lagging strand machinery (which we will now call the primosome in analogy to bacterial terminology) in the absence of a standard replication fork is unknown (50). It seems unlikely that it is the same primosome assembled at the subtelomeric ARSs (50).

Which of the enzymes involved in normal lagging strand DNA replication are required to fill in the complementary strand, how are they recruited to telomeres, and how do they interact with telomerase (50)? Compared to the intensive efforts to study telomerase mechanism, little attention has been aimed at the interaction between telomerase extension and lagging strand fill-in. Several mutants affecting lagging strand polymerases exhibit telomere length deregulation and effects on TPE (1, 15, 41). Mutants lacking *RAD27*, an Okazaki fragment processing enzyme, have long-lived single-stranded TG-tails (48). However, it has been pointed out that these phenotypes could be an indirect effect due to slower replication forks or the inherent instability of repeats in *rad27Δ* mutants (18, 48). One elegant study showed directly that pol α, pol δ, and primase are required for telomere synthesis, whereas pol ε is not (18). A second important study revealed that pol α interacts with Cdc13p, a telomere binding protein (52). Based on this and other recent work (16, 49), it appears that synthesis of the $TG_{1\text{-}3}$-rich strand by telomerase and the $C_{1\text{-}3}A$-rich strand by the lagging-strand enzymes is highly coordinated. Though such coregulation might occur through formation of a large complex of both enzymatic machines at the chromosome ends (21), evidence for such a complex is lacking.

Using a set of assays and specially marked yeast strains previously validated in labs devoted to the study of telomere biology (7, 18, 28, 37, 39, 54), in addition to ChIP (chromatin immunoprecipitation) assays and indirect immunofluorescence, we show directly that Dna2p is both required for telomere biosynthesis and that Dna2p is dynamically sequestered in an apparently large complex at telomeres. This work expands the known repertoire of primosomal proteins needed for C-strand fill-in to processing

enzymes, in addition to the known polymerases. It also answers the question of when and where at least one component of the telomere primosome assembles at telomeres, and the answer is unexpected. Our findings suggest that Dna2p is part of a dynamic telomeric chromatin-based complex that we propose functions not only in telomere replication but also serves as a store for replication and DSB repair proteins, a telomere cap, and perhaps a telomere checkpoint.

MATERIALS AND METHODS

TPE and HML silencing assay. Yeast UCC3505, with the URA3 gene inserted at the left telomere of chromosome VII and UCC3515 (Table 1), with URA3 inserted at HML, were transformed with the pTRP-based plasmid which overexpresses DNA2 under the GAL1,10 promoter, or with pTRP with no insert (54). A 6kb EcoRI-XhoI fragment carrying the entire *DNA2* gene (13, 14) was subcloned into pTRP cleaved with EcoRI-XhoI, placing DNA2 under GAL1,10. Transformants were plated on SD-minus tryptophan plate to select for colonies containing the TRP-bearing plasmids. After 3 days of growth, colonies were restreaked on plates containing 2% galactose and 1.5% raffinose to induce *DNA2* expression. In order to assay the ability to grow in the absence of uracil, samples (5μl) of ten-fold serial dilutions of same numbers of each transformant were spotted onto 2% galactose, 1.5% raffinose minus uracil plates. To control for the number of cells plated, the serial dilutions were also plated on the 2% galactose, 1.5% raffinose plus uracil medium at the same time. Plates were photographed after 4 days of incubation.

Telomere one-hybrid assay. The telomere one-hybrid assay was carried out using strains and plasmids as described (7). For the telomere one-hybrid assay, the *DNA2* ORF (EcoRI-XhoI) was cloned in frame into the EcoRI-XhoI sites of pJG4-5 (7) to make pJG4-5DNA2. When pJG4-5 carries *DNA2*, it expresses a polypeptide that is a fusion of *DNA2* with B42 activation domain, the hemagglutinin (HA) tag, and a nuclear localization signal. B42 is an *E. coli* sequence that activates transcription in yeast. *DNA2* was also cloned in the same vector without the activation domain, as a control.

Isogenic strains (YM701HIS-TEL and YM701HIS-TELsir3Δ (Table 1 and Fig. 2), carrying plasmids expressing the relevant fusion proteins under *GAL1,10* promoter

control, were grown at 30 °C for 2 days in SC liquid medium lacking tryptophan and containing 3% raffinose to avoid glucose repression. The vector, Rap1-B42 and Cdc13-B42 plasmids are described in (7). To screen for expression of *HIS3*, 5 µl of cells was spotted in ten fold dilutions onto 2% galactose-2% raffinose minus histidine plates containing 3-AT (3-aminotriazole), or 2% galactose-2% raffinose plus histidine control plates. It was necessary to use different concentrations of 3-AT to reduce the background of the $His3^+$ phenotype of each transformant, as described in (7). The growth of each transformant was measured after 3 days of incubation on 2% galactose-2% raffinose plus histidine and after 10 days on the same medium minus histidine. The assay was repeated three times on different days.

Indirect immunofluorescence. *DNA2* was fused at its C-terminus to 9 myc-tag peptides and inserted into the chromosome, replacing the endogenous copy of *DNA2*. For this construct, PCR was performed on plasmid pJSVM53H (pRS303-based plasmid containing 9 myc tags adjacent to a *HIS3* marker, gift of R. Deshaies, California Institute of Technology, Pasadena, CA) using an upstream primer, P1, carrying 45 bases of *DNA2* C-terminal coding sequence and 25 bases of myc tag sequence *(AGCGATAAACCTATCATAAAGGAAATTCTACAAGAGTATGAAAGTGAGAACCTGTA CTTCCAGGG)* and a downstream primer P2 containing 25 bases of *HIS3* and 45 bases of the *DNA2 3' UTR:*

(TGTGATAGCTTTCCTGTTATGGAGAAGCTCTTCTTATTCCCCCT GACAGCAGTATAGCGACCAGC). The PCR product was used to transform strain W303*pep4bar1* with selection for growth on histidine resulting in replacement of the endogenous gene under the control of its native promoter, as verified by PCR and

Western blotting with the 9E10 anti-myc tag. The resulting strain showed the same growth rates as the parental strain. This strain, designated DNA2TMTH (Table 1), was used for indirect immunofluorescence.

Indirect immunofluorescence was performed as described (Gotta et al., 1996). Briefly, cells were fixed by incubation in 3.7% formaldehyde. Fixed cells were washed with 1ml YPD containing 1M sorbitol and resuspended in sorbitol buffer. Spheroplasts of fixed cells were obtained by treatment with Zymolyase for 30 min at 30°C. Spheroplasts were deposited on lysine-coated, multi-well slides. Cells were stained sequentially with anti-myc 9E10 primary antibody, then anti-Sir3p antibody, followed by sequential staining with fluorescent secondary donkey anti-mouse and goat anti-rabbit antibody to visualize the signal. Finally, cells were stained with DAPI to reveal DNA. 9E10 anti-myc monoclonal antibody from Babco company and polyclonal antibodies against Sir3p (a kind gift from L. Guarente) were used. Secondary antibodies were goat TRITC-labeled anti-rabbit and donkey FITC-labeled anti-mouse, Jackson Immuno Research, West Grove, PA. As controls, immunofluorescence was performed without primary antibody or without secondary antibody and the possibility of cross-reaction between antibodies was ruled out. Digital images were obtained using a Hamatu digital camera using a Nikon Eclipse TE300 inverted microscope. The camera was controlled by Metaphore image acquisition software (Universal Image Corp., patent 4558302).

Chromatin immunoprecipitation assay (ChIP). The ChIP assay used in this study is based on the methods described by Tanaka et al. *(56)*. Four sets of primers were used for the PCR amplification step; Tel3500.fwd, (TGATTCTGCTTTATCTACTTGCGTTTC), Tel3500.rev (AGAGTAACCATAGCTATTTACAATAGG), Tel300.fwd,

GGATATGTCAAAATTGGATACGCTTATG, Tel-300.rev, CTATAGTTGATTATAGATCCTCAATGTC *(45)*, Cup1-1 (GGCATTGGCACTCATGACCTTCAT), Cup1-2 (TCTTTTCCGCTGAACCGTTCCAGCA) (29), rDNA-1 (CCACCCGATCCCTAGTCGCATAG), and rDNA-2 (GTGTACTGGATTTCCAACGGGGCC). PCR products were separated on a 2% agarose gel and stained with 0.2 μg/ml of ethidium bromide. Gels were photographed and the negatives were scanned and quantitated by densitometry. The DNA2TMTHin Δ strain was constructed in the DNA2TMTH strain (Table 1 and above). The *SIR3* gene was deleted by a one step PCR method using sir3 del fw oligomer (GCAGCCCTTTCATCACCTTCCTTACAGGGGTTTAAGAAAGTTGTTTTG), sir3 del rev (ATTAAGGAATACAGAAGAGACTGCATGTGTACATAGGCATATCTATGGCGG) in the same way as for the DNA2TMTH strain (see above).

Telomerase-mediated telomere addition assay. The telomere addition assay was carried out as described (18). Strain UCC5706 dna2-2 was made in three steps. Strain UCC5706 (a kind gift from D. Gottschling, Fred Hutchinson Cancer Center, Seattle, Washington) was transformed with pSD190 containing *RAD52* to create UCC5706RAD52. Linearized pRS306DNA2C containing the *dna2-2* gene was integrated into UCC5706RAD52 to form UCC5706RAD52dna2-2. To eliminate the pSD190-encoded RAD52 from UCC5706RAD52 dna2-2, the strain was incubated in YPD rich media overnight and was plated on SD minus tryptophan. To confirm the UCC5706 *dna2-2rad52Δ* genotype, temperature sensitivity was checked at 37°C (11).

The assay was performed exactly as described (18). Briefly, cells, wildtype UCC5706 *rad52Δ* and the temperature-sensitive strain, UCC5705 *dna2-2rad52Δ* were pregrown in complete medium lacking lysine (2% glucose), diluted into YEP-2.5% raffinose for four generations, and arrested in the cell cycle with nocodazole at 23°C until 95% of cells showed the appropriate dumbbell cell morphology. Nocodazole was added to a log phase culture from 10 mg/ml stock in DMSO to a final concentration of 10 μg/ml. Cell cycle arrest (2C DNA content) was confirmed by flow cytometry (not shown). For galactose induction, cells were then centrifuged, resuspended in prewarmed YEP-3% galactose, and incubation was continued at 37°C. At various times, cells were harvested, genomic DNA was prepared and digested with enzyme, 0.8% agarose gel electrophoresis was carried out. Hybridization with the ^{32}P labeled *ADE2* gene fragment was carried out after blotting the gel to Nylon membrane (Zeta-probe GT genomic membrane) by vacuum transfer. Telomere sequence addition efficiency at each time point was normalized based on the amount of signal in the band containing *ADE2* from its endogenous chromosomal location (band labeled INT in Fig. 6B) using phosphorimager analysis.

RESULTS

TPE and Dna2p. *dna2* mutations have been reported to affect telomeres in at least three different ways. First, overexpression of 300 amino acids of the N-terminus of *DNA2* reduces TPE (54). Second, telomeres in *dna2* mutants are slightly longer than in wild type, although not as long as in other lagging strand mutants (1, 15, 25, 41). Third, overexpression of *DNA2* leads to rapid appearance of single-stranded $TG_{1\text{-}3}$ tails (48). Dna2p is a candidate for a component of the putative telomeric end replication complex, especially because of the unexpected and as yet unexplained observation that *DNA2* overexpression, just as overexpression of *TLC1*, the RNA component of telomerase, reduces TPE (54).

To increase confidence that the effect of the N-terminal fragment of *DNA2* on TPE had relevance to the function of Dna2p in the cell, we asked whether overexpression of intact *DNA2* also reduces TPE (54). Strain UCC3505 has *URA3* next to the left telomere of chromosome VII (VII-L). The *URA3* gene located at the telomere normally switches between transcriptional states. However, deleting its transcriptional activator gene, *PPR1* causes the telomere-adjacent *URA3* gene to be completely silenced in *UCC3505* and the strain grows poorly on medium lacking uracil. As shown in the serial dilution assay in Fig. 1A, overexpression of *DNA2* increased the efficiency of growth on medium lacking uracil. No difference in the number of Ura^+ colonies was observed between strains containing the vector and *DNA2* constructs on glucose medium, where DNA2 was not overexpressed. Overexpression of the full length *DNA2*, as reported previously for the N-terminal portion of *DNA2* (54), also reduced silencing of *URA3* integrated at the silent mating type locus, *HML*, as evidenced by increased ability to form colonies without

uracil (Fig. 1A). Thus, the full length *DNA2* gene behaves like the N-terminus in reducing silencing, especially TPE.

The same strains used to test the effect of overexpression of *DNA2* on TPE were also used to measure the effect of overproduction on telomere length. As shown in Fig. 1B, overexpression of *DNA2* reduced telomere length. Three independent induction experiments showed the same shortening compared to wildtype. Thus, overproduction of Dna2p reduces both TPE and telomere length.

Telomere one-hybrid assay. To test whether reduction in TPE and telomere length might be a consequence of physical association of Dna2p with telomeric chromatin, we carried out a telomere one-hybrid assay that monitors association of telomeric proteins with telomeres in vivo (7). This assay uses a promoter-defective *HIS3* reporter gene inserted adjacent to $C_{1\text{-}3}A$ tracts at the telomere of chromosome VI (7). This telomere was shown to reflect organization at a natural telomere and to be fully functional (7). Since the *HIS3* gene lacks upstream activation sequences, *HIS3* can only be efficiently activated by binding of a transcriptional activator to the telomere. A similar construct was used to monitor interaction with $C_{1\text{-}3}A$ repeats at an internal position in the chromosome (not shown).

DNA2 was fused to the B42-transcriptional activation domain, a bacterial transcriptional activation domain that functions in yeast. The ability of this Dna2-B42 fusion protein to activate *HIS3* transcription when expressed in yeast was compared to proteins known to interact with telomeric DNA and telomeric chromatin, Rap1p-B42 and Cdc13p-B42, by scoring growth on medium lacking histidine. Dna2p-B42 was able to activate the telomeric $C_{1\text{-}3}A$-*HIS3* reporter gene as efficiently as Rap1p-B42, and activation required

the B42 transcriptional activation domain (Fig. 2A). Since the B42 activation domain is required, it does not appear that overexpression of Dna2p is simply causing titration of factors that silence the *HIS3* gene, but rather that it is bound at the telomeric repeats and is actually activating transcription. [Note that in Fig. 2A the panel on the left constitutes a loading control and shows less growth than in the experiment on the right because these control plates were incubated for only three days while the experimental plates were incubated for ten days, as in the original description of this assay (7)].

Since overexpression of *DNA2* caused derepression of silencing at *HML* as well as at telomeres (Fig. 1A), it seemed plausible that the apparent association of Dna2p with telomeres (Fig. 2A) might require components of silent chromatin shared between *HML* and telomeres. *SIR3* encodes such a protein (53). We therefore asked if the ability to activate transcription in the one-hybrid assay depended on *SIR3* by repeating the assay in an isogenic *sir3Δ* strain, as previously demonstrated for Cdc13 (7). As shown in Fig. 2B, right, the Rap1-B42AD fusion protein activated the telomeric promoter-defective *HIS3* gene, consistent with the fact that Rap1p is known to bind directly to the $C_{1\text{-}3}A$ repeats. Dna2p-B42 failed to express the gene, however, suggesting that binding of Dna2p at telomeres requires a Sir3-dependent chromatin structure (see also Fig. 5D). (Note that the loading control on the left in Fig. 2B was plated on YPGR (yeastextract, peptone, galactose, raffinose), accounting for the higher efficiency of growth compared to controls in Fig. 2A).

Dna2p can be cross-linked to telomeric and subtelomeric regions and localization fluctuates during the cell cycle. As a direct test for association of Dna2p with telomeric DNA, we determined the localization of Dna2p using a ChIP protocol (56). Since Dna2p

is not abundant, in order to detect Dna2p without overproduction, the chromosomal *DNA2* coding sequence was precisely replaced, as described in Materials and Methods, with an epitope tagged *DNA2* gene (*DNA2-myc*) under control of its native promoter. We found no differences in growth rates between cells carrying *DNA2* or *DNA2-myc*. ChIP assays were performed on both asynchronous cells and cells progressing through a synchronous cell cycle. For the synchrony experiments, cells were arrested with α-factor and then released into a synchronous cell cycle. S phase begins about 40 min after release under these conditions (Fig. 3A). Samples of either asynchronous cells (Fig. 3B, right) or synchronized cells at the indicated times after release from α factor (Fig. 3B, left) were crosslinked using formaldehyde and extracts prepared at the indicated times. DNA in the extracts was sonicated to about 500 bp, followed by immunoprecipitation with 9E10 anti-myc antibody. After reversing the crosslinks, quantitative PCR was performed using primers complementary to a subtelomeric region 3500 bp from the right telomere of chromosome VI (Tel3500), and to a region 300 bp from the same telomere (Tel300) (45). The Tel3500 and Tel300 primers amplify unique sequences. Sir3p has been shown to cross-link efficiently to DNA amplified by these probes (45), and yKu has been shown to be associated with similar probes (42). Primers to a centromere proximal region around the *CUP1* gene (29) and to another internal region in the rDNA (*RDN1*) locus were also used (see Materials and Methods). The results shown in Fig. 3B indicate that Dna2-myc binds preferentially to telomeric DNA compared to centromere proximal sequences in G1. Tel300 and Tel3500 are enriched 22-fold and 9.2-fold, respectively, compared to the *CUP1* sequences in the Dna2-myc immunoprecipitates from G1 phase cells. Controls show that *CUP1* probes are amplified with similar efficiency to Tel3500

in extracts (lanes labeled INPUT), even though some strains contain repeated copies of *CUP1*. In S phase, the pattern of DNA associated with Dna2-myc is dramatically reversed (Fig. 3A). The two telomeric PCR products are virtually undetectable in the Dna2-myc precipitates (45 min. time point, cells greater than 90% budded), and the *CUP1* and *RDN1* bands, which should now correspond to replicating DNA, are prominent. The timing of association of Dna2p with *CUP1* and *RDN1* is similar to the timing of association of another replication elongation factor, DNA polymerase ε, with *CYC1*, another centromere proximal locus (43) and serves as an independent marker of S phase. The redistribution of the bulk of Dna2p from Tel3500 and Tel300 to internal DNA sequences at S phase suggests that the Dna2p is sequestered at telomeres in G1, and that this may, in part, reflect storage of Dna2p or a protective capping role.

The preferential telomeric localization pattern is observed again as cells exit S phase or as they enter G2 (75 and 90 min, 100% large budded cells), and remains at a maximum throughout G2. This coincides with the time that telomerase is active in vivo. The final time point in the experiment (105 min. 90% unbudded cells) represents the next G1 phase. The fluctuation in localization is probably not due to changes in expression of *DNA2* during the cell cycle, since its mRNA expression is constant in the cell cycle (17, 55). Western blotting also showed that Dna2 protein was expressed throughout the cell cycle, though there may be a slight reduction in levels during α factor arrest in G1 (data not shown).

It has previously been shown by ChIP assay that Sir3p interacts with both Tel300 and Tel3500 DNA and even with a probe 5800 base pairs internal to the telomere (45). To insure that the ChIP assays for Dna2-myc were valid, extracts were also tested for Sir3p

localization (Fig. 3C). Cells were arrested in our experiment with nocodazole, and arrest was monitored by flow cytometry (not shown). Extracts were crosslinked and immunoprecipitates carried out on parallel samples from the same extract with anti-myc antibody and anti-Sir3 antibody. We found the same enrichment of telomeric DNA compared to *CUP1* in the anti-Dna2-myc immunoprecipitates as we found in cells in G2 phase after release from α factor arrest (compare Fig. 3B and Fig. 3C); and Sir3p showed the same occupancy as Dna2-myc in the nocodazole arrested cells (Fig. 3C). Thus, there is a correlation between the sequences to which Sir3p binds and those to which Dna2-myc binds.

The ChIP experiments shown in Fig. 3B and 3C provide strong evidence that Dna2p is bound to both telomeric and subtelomeric chromatin and that this localization changes with progression through the cell cycle. The presence of Dna2p at telomeres and subtelomeric regions in G1 phase was surprising and implies that sequestration is not solely due to the enzymatic role of Dna2p in Okazaki fragment processing at these chromosomal sites (see below), neither of which replicate in G1 (18, 22, 23, 40). This is the first DNA replication protein that has been found to show preferential subnuclear localization to telomeric chromatin in G1 phase, where there is no DNA replication. The G1 localization is further shown by immunofluorescence studies below (Fig. 5A).

The amount of Dna2p at telomeres in G2 phase is dramatically reduced in a strain lacking *SIR3*. Two results suggested that Sir3p might be required, either directly or indirectly, for the association of Dna2p with telomeres. The one hybrid assay presented in Fig. 2 showed that Dna2p failed to associate with C_{1-3} A repeats in a *sir3Δ* strain. Furthermore, the ChIP assay presented in Fig. 3C showed Sir3p was present on the same

subtelomeric sequence (Tel3500) as Dna2-myc. To test for Sir3p involvement in Dna2p localization, ChIP assays were repeated in an isogenic *sir3Δ* strain (see Materials and Methods). As shown in Fig. 3D, Dna2p localization to telomeres (Tel300) in cells arrested in G2 with nocodazole was indeed severely reduced in a *sir3Δ* strain compared to the isogenic *SIR3* strain shown in Fig. 3C. Association of Dna2-myc with subtelomeric chromatin (Tel3500) was undetectable in the *sir3Δ* □□□□□□. Interestingly, in contrast to the *SIR3* strain, in the *sir3Δ* □□□□□□, *CUP1* sequences are now found in the Dna2-myc immunoprecipitates (compare the *CUP1* bands in Fig. 3C and Fig. 3D). This may suggest that Dna2-myc associates with internal DNA when not sequestered at the telomere, although much further work would be required to verify this. Although the apparent Sir3-dependence of Dna2-myc localization is virtually identical to the Sir3-dependence of yKu localization to telomeric and subtelomeric chromatin (42), it is unexpected for a DNA replication protein. The residual Dna2p associated with Tel300 is consistent with the fact that *DNA2* may be required for their telomerase-dependent replication (see below), which occurs actively in nocodazole-arrested cells (18). The Tel3500 DNA is most likely replicated by standard forks moving out from the subtelomeric ARS, and this replication is likely complete long before cells arrest in nocodazole. Therefore, the Sir3-dependent localization of Dna2p to Tel3500 in G2 is not likely due to the function of Dna2p in subtelomeric replication, which does not require Sir3p. The *SIR3* requirement strongly suggests that the telomeric association of Dna2-myc observed by the ChIP assays is physiologically significant, as well as identifying at least one factor that is important for efficient Dna2p recruitment/and or retention at telomeres.

Localization of Dna2p by indirect immunofluorescence. To obtain an overview of the localization of the entire complement of Dna2p in the cell, we used indirect immunofluorescence microscopy. The same strain, carrying Dna2-myc under the control of its natural promoter, was used for these experiments. Telomeres have been found to cluster in several large foci around the nuclear periphery in yeast (28). Cells from an asynchronous culture were fixed and costained with 9E10 anti-myc antibody, to detect Dna2-myc, and anti-Sir3p antibody, the latter serving as a marker for telomeres (36). Sir3p showed large foci (Fig. 4A) and these were near the nuclear periphery (Fig. 4B), as expected for telomeric foci. Strikingly, Dna2-myc protein also showed a focal staining pattern (Fig. 4A). The merged fluorescence of both anti-myc and anti-Sir3p antibody showed overlap of Dna2-myc foci with Sir3p in almost 80% of the cells (Fig. 4A). Thus, Dna2-myc is abundant at telomeres.

Although 75-80% of the cells showed most of the Dna2-myc in the Sir3-containing foci, 20-25% of the cells clearly showed more dispersed Dna2-myc. The top two cells in Fig. 4B represent the 20% with clearly dispersed Dna2-myc. In the top cell, there appear to be some clumps of Dna2-myc, but there is also a more dispersed Dna2-myc staining, that fails to overlap with Sir3p. The middle cell shows Sir3p foci and even more clearly non-overlapping dispersed Dna2-myc. The bottom cell represents the major class in which the two proteins are almost entirely colocalized. A likely explanation for the heterogeneity, given the results of the ChIP assays, is that the two patterns, focal and diffuse, represent cells at different points in the cell cycle and/or cells in transition (see Fig. 5A).

To insure that the foci overlapped in three dimensions, cells were observed in the confocal microscope. In Fig. 4C, the first picture in each row is an interpretation of the real images of four foci in the first of the six Z-sections of a single cell shown in the six adjacent photographs in the same row. The first row shows Dna2p in green, the second row shows Sir3p in red, and the extent of colocalization of the two proteins is shown in the merged images in the third row (yellow/orange). Three out of four Dna2p foci overlap with Sir3p foci.

Redistribution of Dna2p during the cell cycle as shown by immunofluorescence. To demonstrate that the focal and diffuse staining patterns for Dna2-myc were the result of dynamic localization to telomeres, synchronization studies were carried out. As shown in Fig. 5A, Dna2-myc localized in nuclear foci in G1 phase (peripheral, bright green foci on the blue background of DAPI-stained DNA). This, like the ChIP assays (Fig. 3), suggests that Dna2p is localized to telomeres even at a point in the cell cycle when the telomere is clearly not replicating (18, 40). In contrast to G1 phase, Dna2-myc showed diffuse nuclear staining in S phase, appearing as an aqua stain over almost the entire DAPI stained area. This pattern is different from

that reported for Sir3p, which is found in foci during S phase as well as in the other cell cycle phases (36). The difference in Sir3p and Dna2-myc localization in S phase likely explains our observation that there was not a direct one-to-one correspondence between Sir3p foci and Dna2-myc foci *in every cell* in an asynchronous culture (Fig. 4B). The diffuse staining pattern in S phase is probably not due to an increase in expression of *DNA2* during S phase, since its expression is constant in the cell cycle (17, 55). Furthermore, the ChIP assays confirm the reduction of Dna2p at telomeres during S phase (Fig. 3), and Western blots show the same amounts of Dna2p in the G1, S phase, and G2 phase cells (data not shown). We propose that much of the Dna2p found at telomeres in G1 delocalized during S phase to associate with replication forks for Okazaki fragment processing or to repair DSBs arising due to replication errors (62).

Dna2p redistributes in response to DNA damage. The yKu and Sir proteins are normally enriched in telomeric foci, but redistribute throughout the nucleus in S phase upon treatment of cells with agents that cause DSBs (42, 45). *dna2* mutants are sensitive to such agents (9, 11, 25). Bleomycin causes DSBs by a concerted free radical attack on sugar moieties, preferentially attacking adjacent residues on opposite strands. A serial dilution growth assay shows that *dna2-2* mutant strains are roughly 1000 times more sensitive than wildtype to bleomycin and almost as sensitive as the *rad52Δ*, recombination repair defective control (Fig. 5B).

We examined the localization of Dna2-myc after treatment of wild-type cells with a low level (15 mU/ml) of bleomycin for 3 h. Dna2-myc showed a diffuse staining pattern in all cells, as did Sir3p control (Fig. 5C), rather than the focal pattern shown in Fig. 4A. Like yKu and Sir3p after DNA damage (42, 45), Dna2p and Sir3p do not completely

colocalize in damaged cells, but both are clearly dispersed. Also as in the case of Sir3p and yKu, some foci remain. When a ChIP assay was carried out on extracts prepared from cells treated identically with bleomycin, there was a significant reduction of the amount of Dna2p located at telomeres (Imamura, Choe and Campbell, in preparation). The changes in Dna2-myc focal staining pattern is likely due to relocalization rather than to changes in expression of Dna2p, since Western blotting shows equal amounts of Dna2-myc before and after bleomycin treatment (Fig. 5D). This relocalization of Dna2-myc could be due to some change in telomere structure or to a checkpoint regulated mobilization, like Mec1-dependent yKu and Sir3p redistribution after DNA damage (27, 42). Alternatively, since Dna2p relocalizes in S phase without DNA damage (Fig. 3B and 5A), the Dna2, damage-dependent pattern could be due to an extension of S phase due to engagement of the S phase checkpoint.

***DNA2* functions in telomerase-dependent telomere replication.** *dna2* mutants synthesize a 2C DNA content, but the DNA is fragmented. They are thus deficient in processing of the newly synthesized DNA rather than in synthesis per se (10, 24). To assess whether Dna2p functioned in telomere DNA replication and whether it was required for synthesis and/or processing, a physical assay that allows monitoring of telomere addition at the fully nonpermissive temperature was adopted (Fig. 6A) (18). The experiment measures addition of telomeric repeats onto a chromosomal end generated by induction of the HO endonuclease and terminating in $C_{1\text{-}3}A$ repeats (Fig. 6A). In this assay, *rad52* must be deleted to prevent healing of broken telomeres by recombination. Like many other mutants defective in lagging strand DNA replication, such as *rad27Δ*, most *dna2* alleles show severe growth defects and reduced maximum

permissive temperature when combined with *rad52Δ* mutations. It is possible, however, to isolate viable *dna2-2rad52Δ* mutants. These double mutants grow well at 30°C (9) but become dependent on *RAD52* for survival and fail to grow at 37°C. As shown in Fig. 6B, *dna2-2* shows reduced efficiency of telomere addition at the restrictive temperature. In this figure, the band labeled LM is the product of HO cutting of the fragment labeled PRE, and telomere addition is represented by the SM smear of elongating double-stranded DNA just above LM. Quantitation of the appearance of the SM smear and disappearance of the LM band after normalization by the internal hybridization control (band labeled INT) shows that *dna2* mutants are at least 6 times less efficient in telomere synthesis than wildtype at 240 min. point (Fig. 6C). In *DNA2* wildtype, telomere sequence addition is more efficient than HO cutting, such that LM was rapidly converted to SM. In the *dna2* mutant, telomere sequence addition is less efficient than HO cutting, such that LM accumulated, instead of converting to SM (Fig. 6D). This pattern is striking because formation of LM itself is slower in the *dna2* mutant than in wildtype; that is, HO cutting is less efficient in the *dna2* mutant at nonpermissive temperature than in wildtype (see Fig. 6B, band labeled PRE, and quantitation in Fig. 6D). Thus, LM accumulation in the mutant cannot be due to more rapid production of LM (Fig. 6D), but must be due to failure of telomere sequence addition. [Similar slow digestion with HO was seen in the replication mutants studied previously (18)]. We conclude that *DNA2* is required for telomerase-dependent telomere synthesis, and not just for subsequent processing (see Discussion).

There is some residual elongation of the HO cut end (conversion of LM to SM, Fig. 6) in the *dna2-2* mutant. Since the *DNA2* gene is essential for viability, *dna2-2* mutants, which

are viable at all temperatures (in the presence of *RAD52*), are by definition leaky. Thus, the small amount of synthesis observed may be due to residual *dna2* activity, as was found for pol δ mutants (*pol3*), in the study that originally used this assay (18). However, further work is required to distinguish leakiness from the participation of a backup mechanism.

***dna2* mutations accelerate the senescence of mutants lacking telomerase.** Since the results in Fig. 6 are most simply interpreted as a coordination between telomerase and pol α, pol δ, primase and Dna2p, perhaps akin to the coordination of leading and lagging strands at normal replication forks, we investigated the phenotypes of *dna2* mutants lacking telomerase. *EST2* encodes the catalytic subunit of telomerase. *EST1*, a telomerase RNA binding protein, also affects telomerase activity in vivo, since *est1* mutants show progressive telomere shortening (32). To test the interaction between *DNA2* and telomerase function, strains heterozygous for both *DNA2* and *EST2* or *DNA2* and *EST1* were sporulated and tetrads dissected. When otherwise isogenic *dna2-2EST2/DNA2est2Δ* or *EST2/est2Δ* heterozygotes were sporulated, the *dna2-2est2Δ* and *est2Δ* spores germinated efficiently (see legend to Fig. 7A and Table 1). When the spores were restreaked, however, there was a large reduction in viability in the *dna2-2est2Δ* compared to the *est2Δ* colonies (Fig. 7A). The same was true for spores with *dna2-2estΔ* genotype compared to *est1Δ*; that is a much smaller percentage of the double mutant cells remained viable after the germinating spore grew into a colony and was restreaked (data not shown). This suggests that the double mutants senesce more rapidly than the single *est* mutants (Fig. 7A).

Dna2p may participate in telomerase-independent telomere elongation. Although most cells in the double mutants die, survivors do appear. Telomere maintenance was monitored in both the *dna2-2est1Δ* and *est1Δ* strains as they senesced. Although initially the small percentage of cells remaining viable in the *dna2-2est1Δ* spores grew slowly, the *dna2-2est1Δ* survivors showed much larger colony morphology than the *est1Δ* survivors after only 60 generations (data not shown). This suggested that so-called type II survivors, which have a growth advantage over type I survivors, might arise more rapidly in the *dna2-2est1Δ* in the *est1Δ* mutant (39, 59). Type I survivors arise through *RAD51*-dependent homologous recombination between Y' elements, whereas type II survivors acquire very long $C_{1\text{-}3}A$ tracts, due to some as yet uncharacterized, *RAD51*-independent, *RAD50*-dependent mechanism (37, 39, 58, 59). Type I telomeres have multiple, tandem copies of Y' elements terminating in a short segment of $C_{1\text{-}3}A$ tracts, and have lengths slightly longer than normal telomeres. Type II telomeres arise from very short telomeres and contain up 12 kb of $C_{1\text{-}3}A$ tracts. This is the type of survivor seen in alternative methods of maintinaing telomeres (ALT) in mammalian tumor cells (8). Single colonies arising from the first restreak of the germinated *est1Δ* and *dna2-2est1Δ* spores were serially passaged, and the structure of the telomeres monitored during this outgrowth (39). DNA from survivors isolated after various numbers of passages was digested with XhoI, electrophoresed, blotted and probed with a $C_{1\text{-}3}A$ repeat probe (Fig. 7B). After the first passage, telomeres seemed to have shortened in both *dna2-2est1Δ* and *est1Δ* to a similar degree. However, patterns characteristic of fast-growing type II survivors appeared much earlier in the double mutant (two passages or about 40 generations, Fig. 7B, lanes 7 and 9) than in the single *est1Δ* mutant (four passages, Fig. 7B, lane 10). In lanes 7 and 9, the

dna2-2est1Δ double mutant, a smear of new higher molecular weight bands appears concomitant with a decrease in intensity in the broad telomeric XhoI bands characteristic of the early survivors or wild type (marked Y' in lanes 2-5). In lanes 6 and 8, the *est1Δ* mutant after the same number of passages, neither the disappearance of the major XhoI band nor the smear of longer bands is seen. The pattern in lane 7 and 9, *dna2-2est1Δ*, corresponds to that described as Type II [see Fig. 1B, lane 5, in Lundblad and Blackburn (1993) and Le et al. (1999)]. In Fig. 7B, lanes 4 and 6, *est1Δ*, the faint band running just above the 1 kb marker may represent type I survivors that have acquired a Y' element in the *est1Δ* strain. Since type II grow more rapidly than type I, type II eventually appear in *est1Δ* and predominate in the later passages from both *dna2-2est Δ* and in *est1Δ* colonies (lanes 10-15). Lanes 2 and 3 contain controls showing that telomeres are more heterogeneous in length in *dna2*-2 than in an isogenic *DNA2* strain, though not exclusively longer as reported previously in a different genetic background (25). Premature senescence and early appearance of type II survivors is also seen in *rad51tlc1Δ* double mutants (37).

DISCUSSION

Roles of Dna2p localization and relocalization in telomere replication. Although dynamic association/disassociation of telomere factors and their relation to the functions of telomeres is an active area of current research (16, 49), the studies generally fail to directly address the interaction of telomerase with the putative telomeric primosome. Yet, given the evidence, albeit indirect, that there is coordination of telomerase with components of the lagging strand apparatus, a full understanding of the end-replication problem will only be possible by understanding lagging strand functions at telomeres as well as telomerase and its components (21). Our localization results are just the beginning, but the details are surprising and suggest much more complex functions for replication proteins than heretofore proposed. We have established that, in addition to lagging strand DNA polymerases, Dna2p, a processing enzyme, has a function in de novo telomere synthesis (Fig. 6 and 7). What is unanticipated is that Dna2p does not appear to function solely in processing, but that it is required for synthesis itself (Fig. 6). (We reject the alternative idea that 100 bp Okazaki fragments are made and destroyed as inconsistent with previous work on discontinuous synthesis). This is the first direct evidence that an entire primosome, that is one containing both synthesis and maturation functions, is essential for proper end replication. In support of a telomeric primosome complex containing Dna2p is that *dna2* mutants are synthetically lethal with *ctf4* mutants. Ctf4p is a pol α binding protein and ctf4 mutants dramatically reduce the elongated telomeres observed in *pol1* mutants (25). We propose that, as in bacteria, there may be at

least two ways to assemble a eukaryotic primosome: one mediated by Orc-dependent assembly of primosomes at unwound replication origins and a second mediated perhaps by the telomerase machine at single-stranded chromosomal termini.

The data in Fig. 6 show not only that Dna2p is required for synthesizing a double-stranded telomere. They also imply that Dna2p must interact with or is dependent on previous action of telomerase in order to synthesize this double strand (18). The fact that *dna2-2* mutants show slightly longer telomeres than wildtype (25), yet are deficient in the telomere elongation assay (this work), suggests that completion of the lagging strand involving Dna2p at telomeres may require tight co-ordination of the lagging strand replisome with telomerase activity. The synergistic effect on senescence of introducing the *dna2-2* mutation into an *est2Δ* or *est1Δ* mutant (Fig. 7A) is also consistent with integration of leading strand synthesis by telomerase and lagging strand maturation by a primosome (if one interprets synthetic lethality as indicating interaction). One might envision that, just as the standard primosome coordinates with the leading strand replication machinery at a conventional fork, a similar primosome coordinates with telomerase for concomitant 3' extension of the G-rich strand and 5' to 3' fill-in of the C-rich strand.

A role in telomere capping? It was during our probing of the spatial and temporal pattern of Dna2p interaction with telomeres, that we encountered evidence suggesting that Dna2p played additional roles at telomeres. We propose that one of these is in the telomere capping or end protection process. First, the relative fraction of the cellular Dna2p sequestered at telomeres (Figs. 4) is unexpectedly large for the synthesis of what would be anticipated to constitute a small fraction of the total number of Okazaki

fragments. Second, the timing of association/dissociation is unexpected for a protein having a role exclusively in end-replication. Namely, Dna2p is at telomeres in G1 phase (Fig. 3, Fig. 5), when there is no DNA replication in any part of the chromosome (18, 40). At this time, telomerase is present and catalytically active, a state that has been shown to have a protective effect on telomeres (44), and transcriptional activators cannot activate transcription at telomeres, suggesting a closed structure (21). Elsewhere in the chromosome, ORC, Cdc6 and the Mcms, replication proteins that will later recruit the replication fork primosome, are associating with ARSs. Dna2p (Fig. 3), however, like other elongation factors, is undetectable in the bulk chromatin (2, 56, 57). This timing and several additional findings in our work lead to the interpretation that the G1 telomeric Dna2p may be involved capping of the telomere. First, while localization of Dna2p at $C_{1-3}A/TG_{1-3}$ repeats in G2 might be expected, since their terminal portion is replicated by telomerase, enrichment in G1 is unexplained. Second, enrichment in sequences 3500 bp away from the telomere in G1 is unexpected since this DNA is replicated by the standard replication fork, which is not present in G1. Association with subtelomeric chromatin probably accounts for the effects of overproduction on TPE (Fig. 1). TPE in turn may reflect telomere capping, since several of the same proteins affect both (6). Perhaps an imbalance in one putative component, Dna2p, may lead to disruption of the capping/silencing complexes. Our observation that telomeres shorten when Dna2p is overproduced (Fig. 1B) is also more consistent with disruption of a protective cap than with an effect of overproduction on Okazaki fragments. A capping role is also supported by the fact that the phenotype of *dna2-2est1Δ* (Fig. 7) mutant is the same as yKu*/tlc1Δ*

double mutants (31). yKu is a well known component of the telomere cap in yeast (44 for review).

We were at first surprised at the dramatic change in Dna2p localization at the G1/ S phase transition. Instead of associating with telomeres for their replication at this time, Dna2p spreads out throughout the entire nucleus (Fig. 3 and 5) and is now found associated with internal sequences replicating from activated ARSs (Fig. 3). In fact, Dna2p is not even detectable at the telomere in the ChIP assay at the first S phase time point, when it is already associated with Cup1 and the rDNA. We propose now that this relocalization is associated with transient uncapping of telomeres, and preparation for their replication later in the cell cycle. This may represent the "judiciously regulated degree of uncapping" proposed by Blackburn during which the telomere has to actually transiently resemble a break for telomerase to act (6). A role for Dna2p in capping would also explain why Sir3p, required for TPE but not for DNA replication, is apparently required for Dna2p association with telomeres (Fig. 2B and Fig. 3). The Sir3p requirement for association with subtelomeric chromatin, in addition to the ends, would be especially surprising if the only role of Dna2p were for DNA replication, since these sequences should obtain their replicative complement of Dna2p from standard replication forks and not need Sir3p to recruit Dna2p for Okazaki fragment processing.

An alternative ad hoc model is that Dna2p does not function in capping, but is merely stored at telomeres and that its release from storage at telomeres is an S phase regulatory mechanism. It is noteworthy that Dna2p is an unusual DNA replication protein in that *DNA2* contains no MCB cell cycle regulated upstream promoter element and accordingly is not transcriptionally regulated in the cell cycle (55). Thus, Dna2p may be regulated

differently from other replication proteins. There is, however, no precedent for such a model.

In late S phase and G2 phase, Dna2p is recruited again to telomeres. This correlates with the time at which Dna2p is required for telomerase-dependent telomere elongation (Fig. 6) and when subtelomeric regions replicate (22, 60). The large amount of Dna2p now associated with teleomeres might also have a capping role in stabilizing the newly formed telomeres. Supporting this additional role, again Sir3p is required for localization in G2, for at least some of the Dna2p (Fig. 3C).

A checkpoint role for Dna2p at telomeres-unifying capping, replication and repair. Sir proteins and yKu can relocalize from telomeres to DSBs elsewhere in the chromosome (42, 45, 53). The change in the Dna2-myc staining pattern in nuclei after DNA damage (Fig. 5) could analogously suggest that Dna2p is stored at telomeres and targeted away from telomeres to the sites of damage. If the only role for Dna2p at telomeres were C-rich strand fill-in, then relocalization after DNA damage would not be necessary. Release could occur in several different ways. Damage at telomeres might lead to an alteration of telomeric structure that releases Dna2p, or the DNA damage checkpoint might induce relocalization, as has been shown for Sir3p and yKu (26, 42, 45). It is not yet known if *RAD9* or *MEC1* is required for relocalization of Dna2p, as they are for Sir3p and yKu (42, 45). This response to damage may also reflect the telomerase-independent role of Dna2p in telomerase biogenesis (Fig. 6). Interpretation of the redistribution seen in our damage experiments will require further work, however, since the effect of DNA damage is superimposed on the cell cycle distribution seen in undamaged cells.

An appealing alternative model for telomeric sequestration of proteins suggests that assembly of Sir proteins, yKu, and now Dna2p, at telomeres signals the completion of both DNA replication and repair of DSBs through a telomere chromatin checkpoint (27). When these proteins are free in the nucleus, it triggers a checkpoint to block entry into mitosis until DNA replication and repair are complete and the proteins return to telomeric chromatin, releasing cells from the checkpoint. The involvement of Dna2p in a checkpoint has been proposed to account for the fact that *dna2mec1* and *dna2rad9* double mutants are viable, though they fail to arrest mitosis (24, 25). In a *mec1* mutant, Dna2p might, like yKu and the Sir proteins, also remain associated with telomeres allowing passage through mitosis and a second chance to repair damage in the next cell cycle.

In summary, the localization study, supported by genetics and biochemistry, suggest a dual role for Dna2p at telomeres. We provide the first evidence of when and where the lagging strand proteins associate with telomeres. Taken together with the demonstration by others that pol α is found associated with Cdc13p, a telomere binding protein, it also suggests that a primosome complex forms that may account for coordination between telomerase and lagging strand maturation. In addition, we provide evidence for a novel capping/checkpoint function for a lagging strand DNA replication protein.

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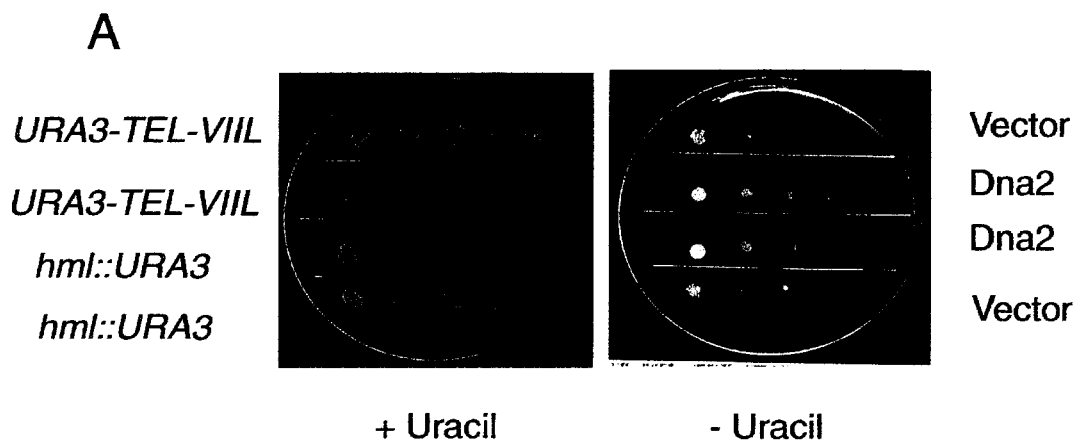
Table 1		
Strains	Genotype	References
YM701HIS-Tel	*MAT a, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, tyr1, adh4::URA3-TG(1-3)-promoter-defective HIS, 20kb Δof DNA Distal to adh4*	*(7)*
YM701HIS-INT	*MAT a, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, tyr1, adh4::URA3 -promoter-defective HIS*	(7)
YM701HIS-TELsir3Δ	*MAT a, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, tyr1, adh4::URA3-TG(1-3)-promoter-defective HIS, 20kb Δof DNA sir3::LYS2 Distal to adh4*	(7)
UCC3505	*MAT a, ade2-101, his3-200, leu2-1, lys2-801, trp1-63, ura3-52, ppr1::HIS3, adh4::URA3-TEL-VIIL, DIA5-1*	(54)
UCC3515	*MAT α, ade2-101, his3-200, leu2-1, lys2-801, trp1-63, ura3-52, hml::URA3*	(54)
UCC5706	*MAT a-inc, ura3-52, lys2-801, ade2-101 ochre, trp1-63, his3-200, leu2-1::GAL1-HO-LEU2, TELVIIL::ADE2-TG(1-3)-Hosite-LYS2, rad52::HISG*	(18)
UCC5706dna2-2	*MAT a-inc, ura3-52, lys2-801, ade2-101 ochre, trp1-63, his3-200, leu2-1::GAL1-HO-LEU2, TELVIIL::ADE2-TG(1-3)-Hosite-LYS2, rad52::HISG, dna2::dna2-2-URA3*	This work
est2	*MAT a, leu2Δ0, his3Δ1, met15Δ, ura3Δ0, est2::KANMX4*	Research Genetics
est2dna2-2	*MAT a, leu2Δ0, his3Δ1, met15Δ, ura3Δ0, est2::KANMX4, dna2-2sol3Δ::LEU2*	This work
TF1087-10-2	*MAT α trp1 leu2 ura3 his3 dna2-2 sol3-Δ (::LEU2)*	(25)
TF7610-2-1	*MAT α trp1 leu2 ura3 his3 DNA2 sol3-Δ(::LEU2)*	(25)
est1	*MAT a, leu2Δ0, his3Δ1, met15Δ, ura3Δ0, est1::KANMX4*	Research Genetics
JCYrad52	*MAT a, rad52-8Δ::TRP1, ura3, trp1, leu2, his3, gal2*	(11)
DNA2TMTH	*MAT a, can1-100, leu2-3,-112, his3-11, -15, trp1-1, ura3-1, ade2-1, pep4::TRP1, bar1::LEU2, dna2::DNA2-TEV-9MYC-HIS3*	This work
DNA2TMTH Sir3 Δ	*MAT a, can1-100, leu2-3,-112, his3-11, -15, trp1-1, ura3-1, ade2-1, pep4::TRP1, bar1::LEU2, dna2::DNA2-TEV-9MYC-HIS3 sir3::KAN^R*	This work
est1dna2-2	*MAT a, leu2Δ0, his3Δ1, met15Δ, ura3Δ0, est1::KANMX4, dna2-2sol3Δ::LEU2*	This work

Figure legends

Figure 1A. TPE is reduced by overexpression of *DNA2*. UCC3505 and UCC3515, containing *URA3* at the telomere or at *HML* as described in Materials and Methods were transformed with a plasmid expressing *DNA2* under *GAL1,10* control or with the plasmid vector alone as control. Tenfold serial dilutions of the indicated transformants were plated on medium containing galactose, to induce *DNA2*, and plus or minus uracil, to monitor expression of the *URA3* gene.

Figure 1B. Telomere length is reduced by overexpression of *DNA2*. Dna2p was overproduced in strain W303*RAD5* using a plasmid with *DNA2* under control of the *GAL1,10* promoter as described in Materials and Methods. The same strain containing vector alone was used as a control. A single colony was picked, and after three restreaks (about 100 generations) on YPGal plates, telomere length was determined as described (48). DNA was digested with XhoI, which cleaves near the telomere proximal end of Y' repeats, leaving primarily tracts of $C_{1\text{-}3}A$ repeats that migrate in agarose gels around the size of the 1 kb marker. Telomere length was assessed by gel electrophoresis and Southern blotting using hybridization with a ^{32}P labeled 22-mer of the telomere sequence 5'-CCCACCACACACACCCACACCC-3' (referred to as the CA oligonucleotide) as described in (39). Each lane contains equal amounts of DNA. Lane 1, strain carrying plasmid lacking *DNA2* insert; lane 2, overexpression of *GAL-DNA2*. The diffuse band around 1 kb represents the Y' telomeres.


A
URA3-TEL-VIIL
URA3-TEL-VIIL
hml::URA3
hml::URA3
Vector
Dna2
Dna2
Vector
+ Uracil
- Uracil

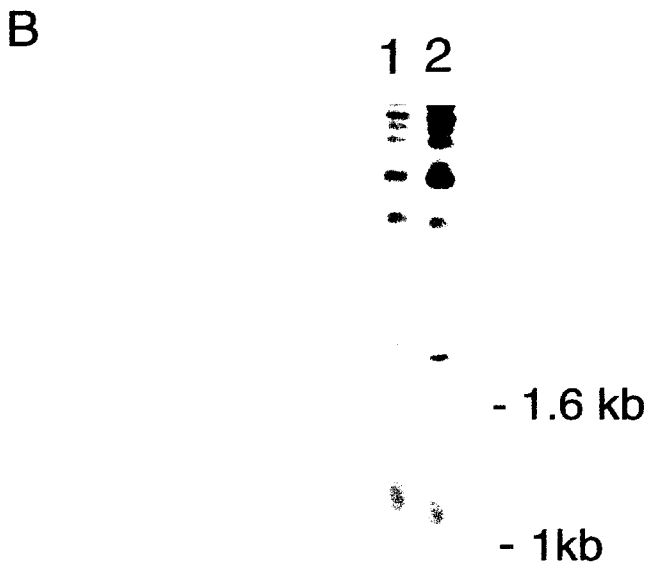

B
1 2
- 1.6 kb
- 1kb

Figure 1

Figure 2. Telomere one-hybrid assay of *DNA2*. The genes indicated were fused to the bacterial B42 transcriptional activation domain as described in Materials and Methods and were overexpressed under the *GAL1,10* promoter. The promoterless *HIS3* reporter gene was integrated at the telomere (A) (7). In (B), the *HIS3* reporter was integrated at the telomere in an isogenic strain carrying *sir3Δ*. (See Materials and Methods and Table 1 for strain descriptions). The experimental results are shown in the panels on the right in (A) and (B). On the left are loading controls. The loading control in panel (A) was plated on Galactose-Raffinose plus histidine and in panel (B) on YPGR (YP plus galactose and raffinose).

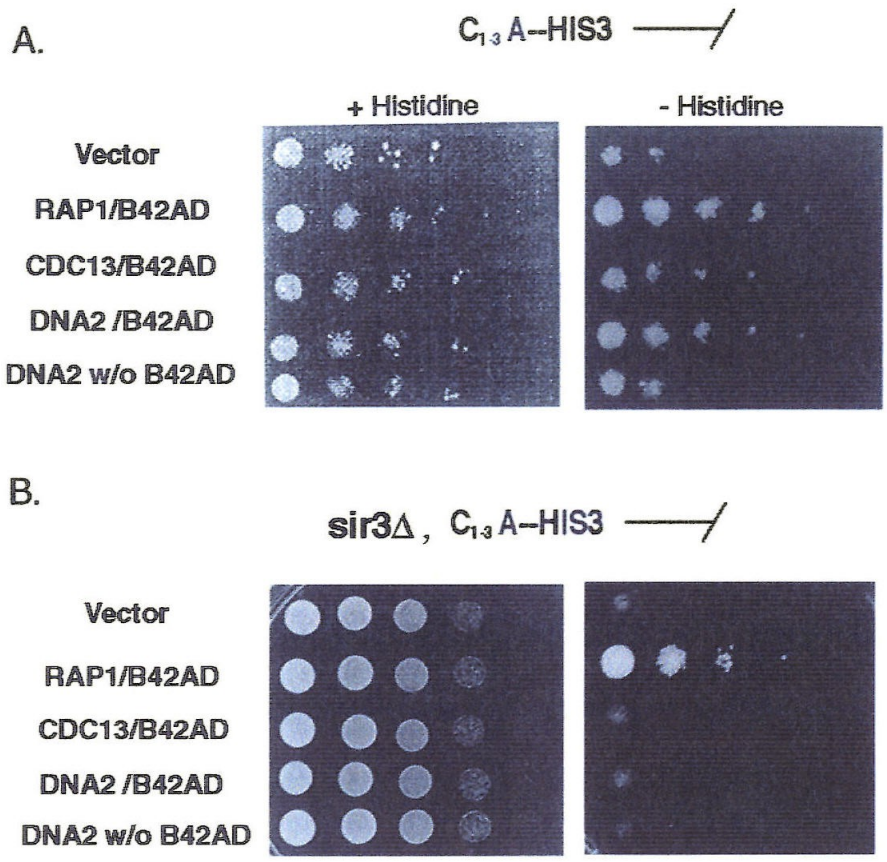

A.
C_{13}A--HIS3
+ Histidine
- Histidine
Vector
RAP1/B42AD
CDC13/B42AD
DNA2 /B42AD
DNA2 w/o B42AD
B.
sir3Δ , C_{13}A--HIS3
Vector
RAP1/B42AD
CDC13/B42AD
DNA2 /B42AD
DNA2 w/o B42AD

Figure 2

Figure 3. Chromatin immunoprecipitation assay of Dna2p localization. (A) Flow cytometric analysis of experiment shown in Fig. 3B. PI (propidium iodide) is a measure of DNA content. (B) Cross-linking of Dna2p to various DNA sequences in asynchronous (right two lanes) and in synchronized cells (panel on the left) as indicated. An experiment using Dna2p-myc immunoprecipitates is shown. Cells were synchronized as in Materials and Methods and shown in Fig. 3A. ChIP assays were performed on samples collected at the time indicated after release from pheromone arrest. The regions amplified by the primers are described in the text. Tel refers to telomeric and subtelomeric primers, while *CUP1* and rDNA (*RDN1*) primers are non-telomeric sequences. The Tel300 primers give a PCR product identical in length to the *CUP1* PCR product and therefore represents a separate PCR reaction, as does the rDNA PCR series. PCR reactions were in the linear range, as determined by monitoring different numbers of cycles. INPUT indicates that PCR reactions were preformed on the extracts from which the immunoprecipitates were prepared, using the primers indicated, providing a loading control as well as a control that the primers used amplified the respective DNAs equally. The lanes at the left and right of each strip contain the size markers. For asynchronous cells, only Tel300 primers were used for the PCR.

(C) Binding of both Sir3p and DNA2-myc protein to telomeric sequences in nocodazole-arrested (G2/M) phase cells. A Dna2-myc tagged (strain DNA2TMTH) was arrested with nocodazole as described in Materials and Methods. ChIP assays were performed using anti-myc to localize Dna2-myc and anti-Sir3 antibody ofr Sir3p. The left lanes

represent the Dna2-myc immunoprecipitates. Right two lanes represent a Sir3p-immunoprecipitate from the same extracts. Primers used to generate the PCR products are as indicated. INPUT is PCR using the extract from which the IP was prepared. Lanes labeled untagged DNA2 represent anti-myc immunoprecipitates from strains synchronized in the same way but lacking a Dna2-myc protein, as a control for the specificity of the antibody.

(D) Localization of Dna2p from G2 phase DNA2TMTH*sir3Δ* cells (see Table 1*)*. The chromatin immunoprecipitation experiment was identical to that in Fig. 3C. The *sir3Δ* strain used was isogenic with the strain used in (C) and was constructed by standard PCR techniques (see Material and Methods and Table 1).

A

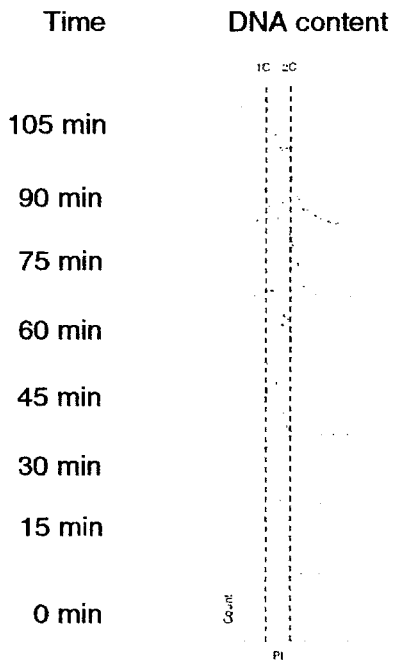

Time
DNA content
105 min
90 min
75 min
60 min
45 min
30 min
15 min
0 min
1C
2C
Count
PI

Figure 3

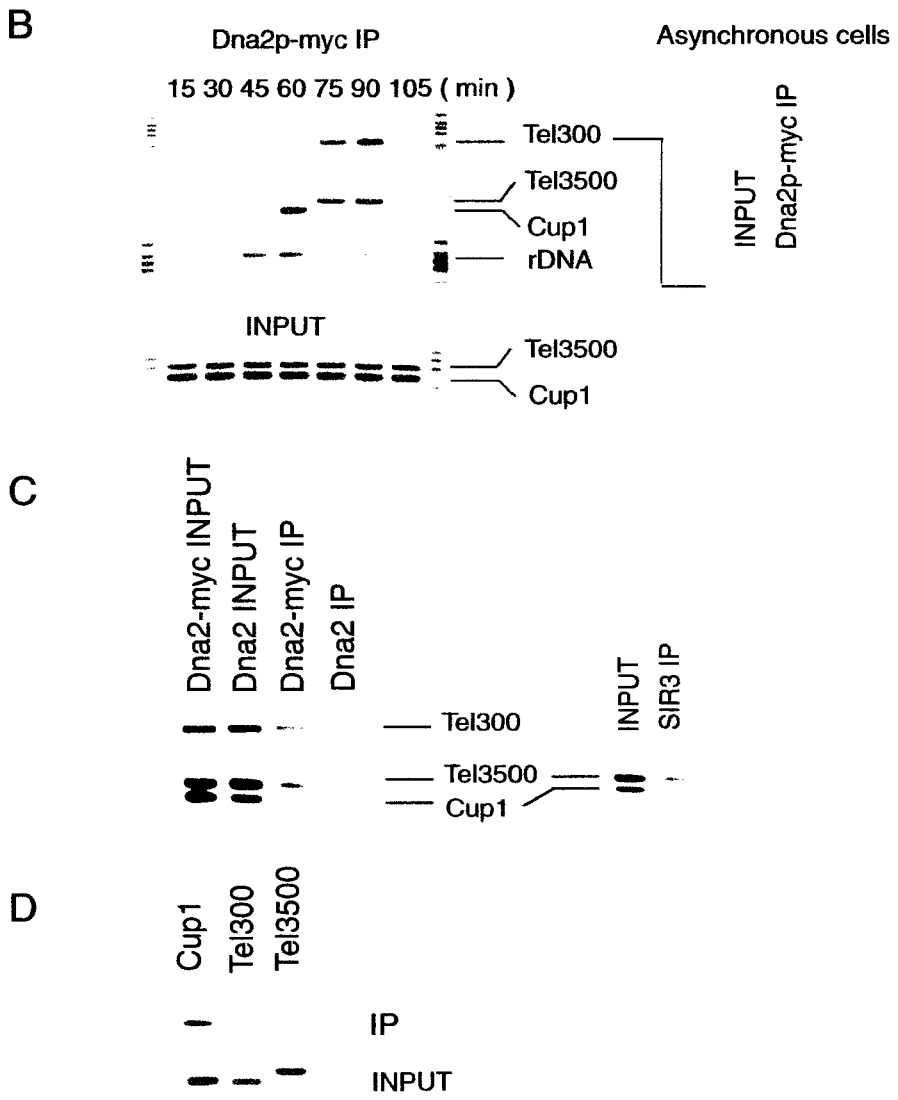

B
Dna2p-myc IP
Asynchronous cells
15 30 45 60 75 90 105 (min)
Tel300
Tel3500
Cup1
rDNA
INPUT
Dna2p-myc IP
INPUT
Tel3500
Cup1
C
Dna2-myc INPUT
Dna2 INPUT
Dna2-myc IP
Dna2 IP
Tel300
Tel3500
Cup1
INPUT
SIR3 IP
D
Cup1
Tel300
Tel3500
IP
INPUT

Figure 3

Figure 4. Dna2p is concentrated in telomeric domains. Indirect immunofluorescence was carried out as described in Materials and Methods. (A) DNA in nuclei was stained with DAPI and is shown in blue. Staining of Sir3p (red) and Dna2-myc (green) and costaining (yellow) are as indicated. (B) Left column, black and white images of anti-myc stained cells. Center column, same cells showing the anti-Sir3p stain. Right column, high-resolution color image of the merged anti-Sir3p and anti-myc stained images of three different cells. Yellow indicates colocalization. The bottom cell represents 80% of the cells in the culture. The remaining 20% appear similar to the top and middle cells, indicated by brackets. About 50 cells were evaluated. (C) Confocal microscopy of six sections through a single cell stained with Sir3p antibody (red), dna2-myc (green) and the bottom row shows the merged images in each section of the Z stack shown. Location of foci within the nucleus are diagrammed to the left.

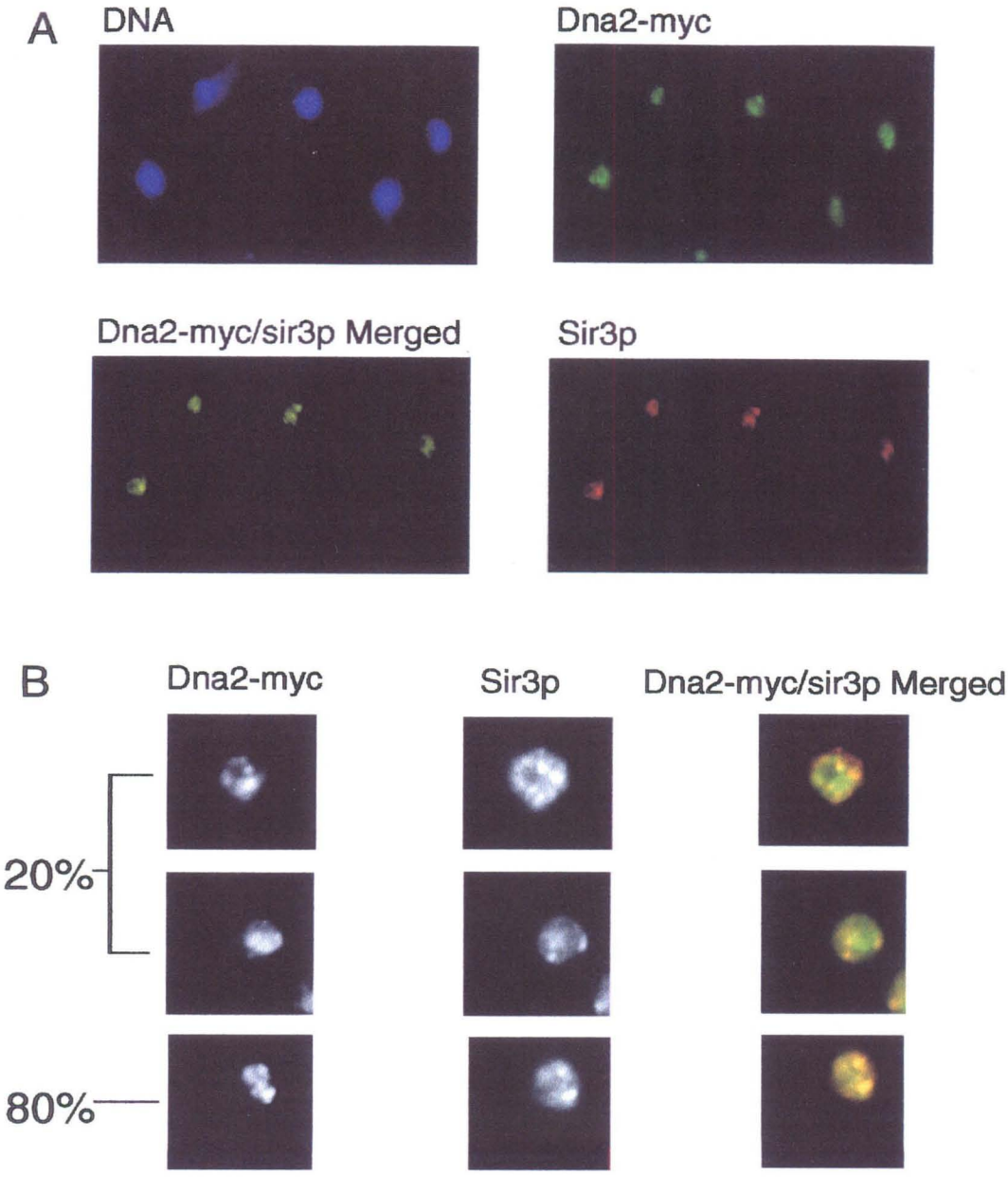

A
DNA
Dna2-myc
Dna2-myc/sir3p Merged
Sir3p
B
Dna2-myc
Sir3p
Dna2-myc/sir3p Merged
20%
80%

Figure 4

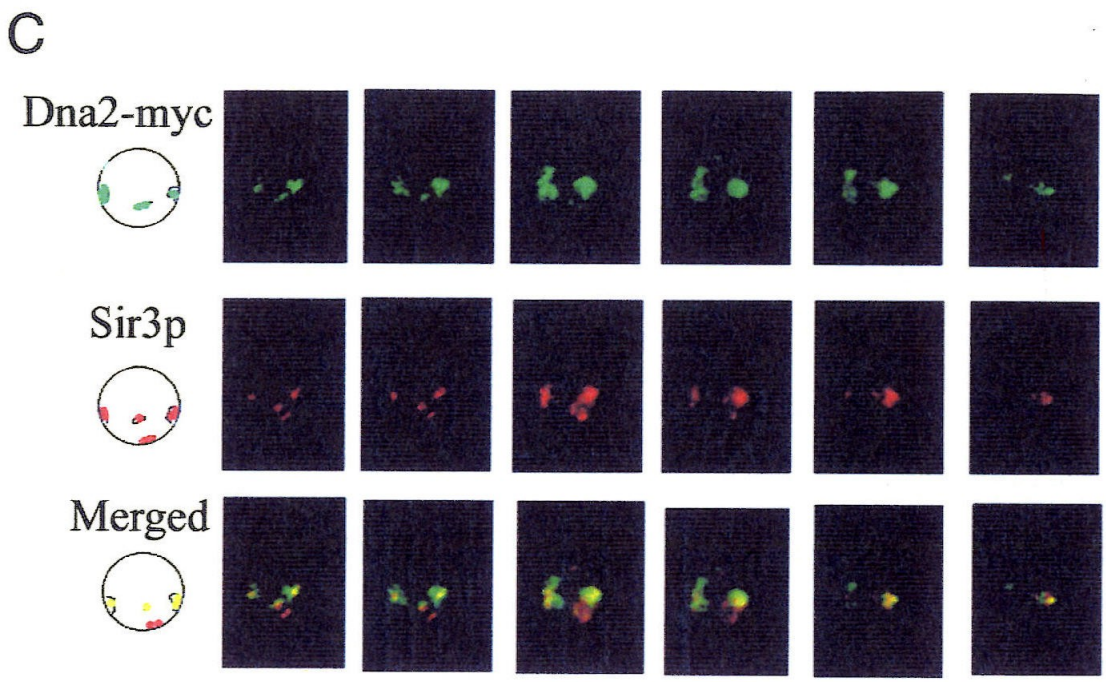


Figure 4

Figure 5. Dna2p localization is dynamic. (A) Dna2p relocalizes in the nucleus during the cell cycle. Cells arrested by α-factor were released in YPD media and were harvested at various times after release as described in Materials and Methods. Cells in G1, S, and G2/M cells were identified by flow cyotmetry analysis of each sample, as indicated; and Dna2-myc localization was examined by indirect immunofluorescence with anti-myc antibody (green stain) at the cell cycle stage indicated. Cells were costained with DAPI for DNA (blue). Preparation of cells for flow cytometry was performed as described (5).

(B) Bleomycin sensitivity of strain TF1087-10-*2dna2-2* and TF7610-2-1*DNA2* and JCY*rad52Δ* (see Table 1). Tenfold serial dilutions of log phase cultures were plated on YPD medium with or without 15 mU/ml bleomycin.

(C) Indirect immunofluorescence after treatment with bleomycin- Sir3p (red) and Dna2-myc (green), merged, yellow indicates colocalization. Co-staining and visualization were carried out as described in Materials and Methods and Fig. 3.

(D) Dna2p levels do not rise after DNA damage. Extracts were prepared from untreated or bleomycin treated cells and the level of Dna2-myc was determined by Western blotting as described previously (14). 2X refers to loading of twice as much extract as in the 1X lane, to insure the blot was in a linear range of Dna2p concentration. A loading control using Cdc28 and anti-Cdc28 antibody showed equal loading of the 1X lanes from bleomycin-treated and untreated cells (not shown).

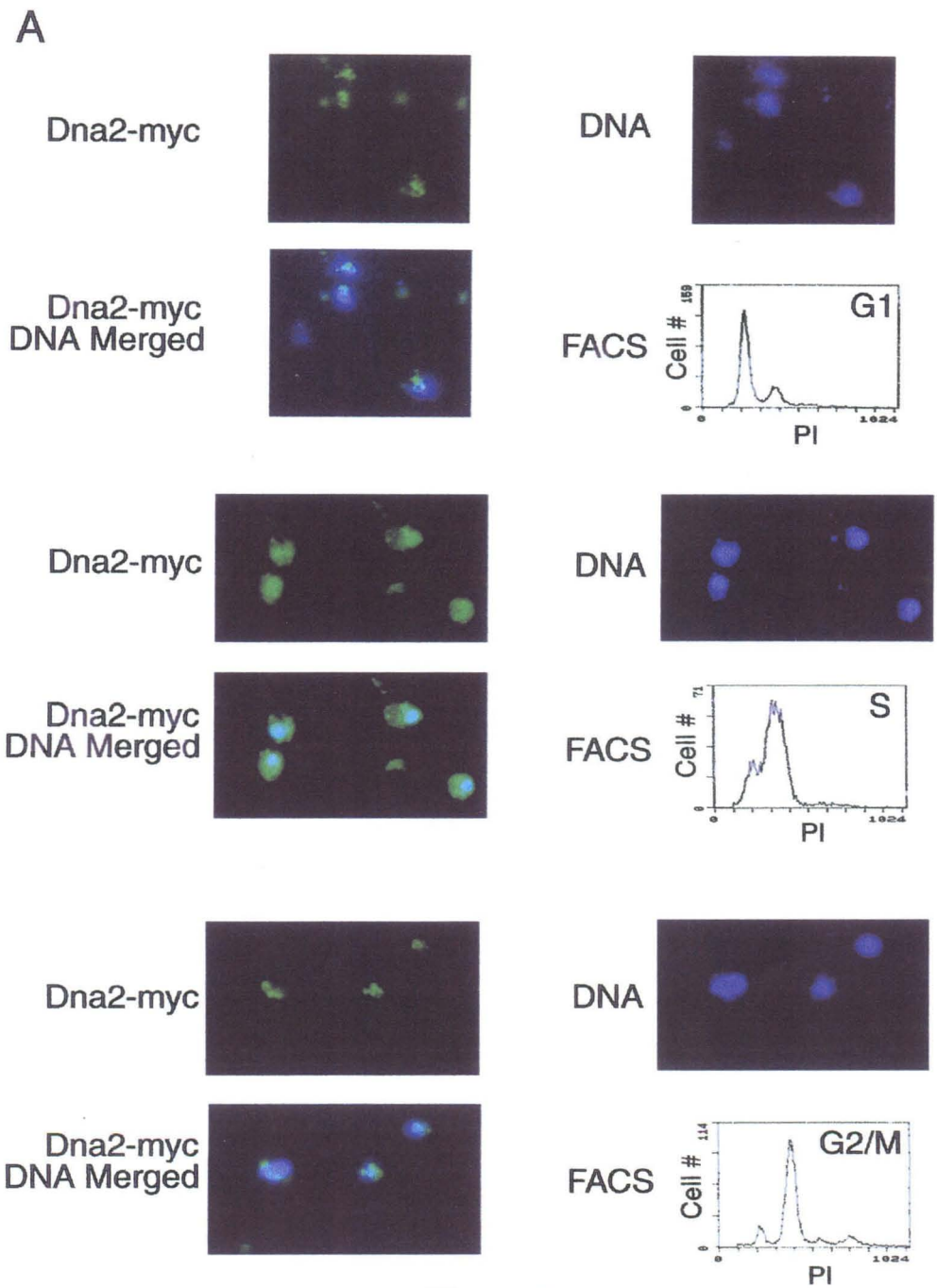

A
Dna2-myc
DNA
Dna2-myc
DNA Merged
FACS
G1
Cell #
PI
Dna2-myc
DNA
Dna2-myc
DNA Merged
FACS
S
Cell #
PI
Dna2-myc
DNA
Dna2-myc
DNA Merged
FACS
G2/M
Cell #
PI

Figure 5

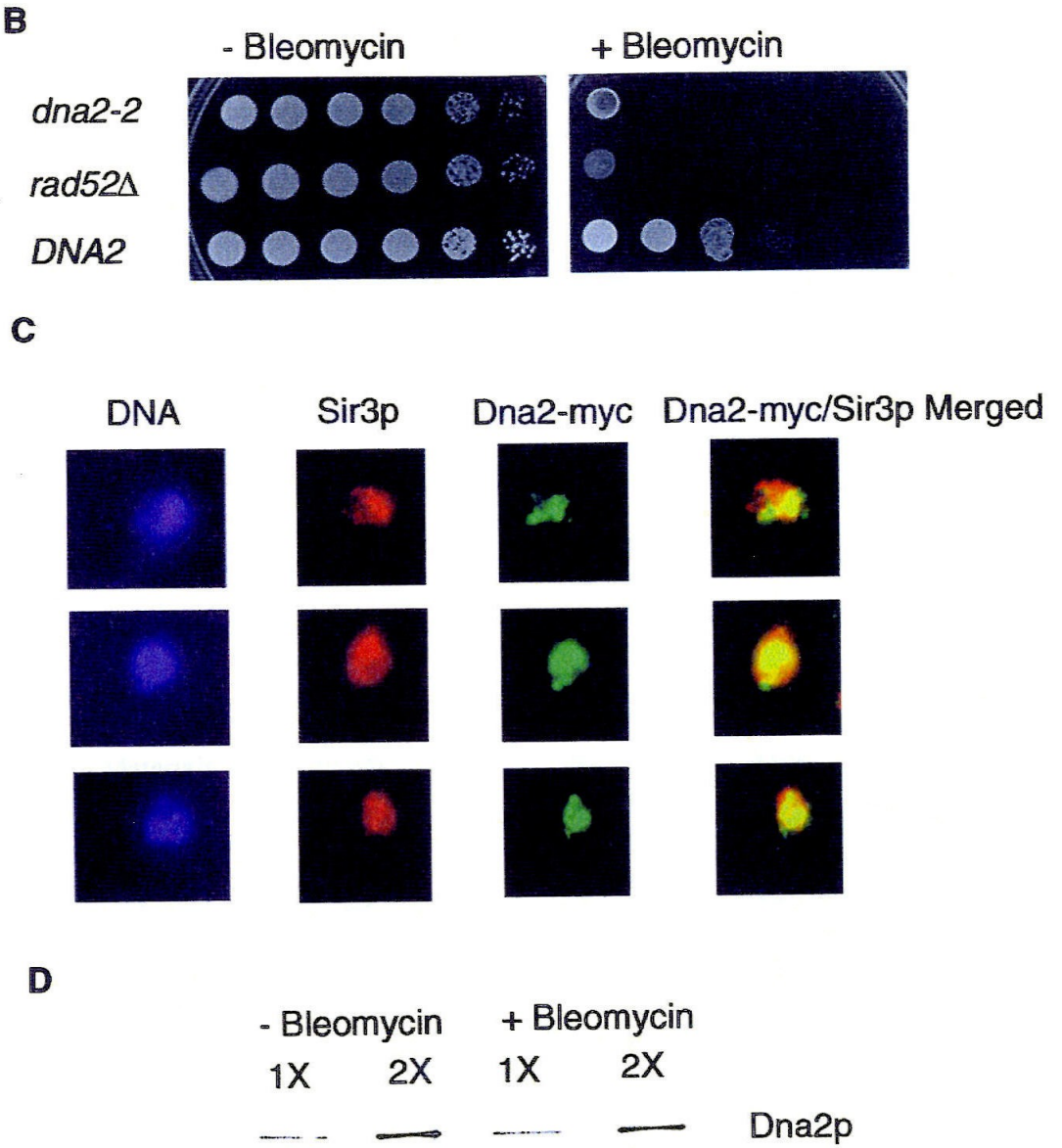

B
- Bleomycin
+ Bleomycin
dna2-2
rad52Δ
DNA2
C
DNA
Sir3p
Dna2-myc
Dna2-myc/Sir3p Merged
D
- Bleomycin
+ Bleomycin
1X
2X
1X
2X
Dna2p

Figure 5

Figure 6. Dna2p plays a role in telomerase-dependent telomere replication. (A) Schematic representation of the telomere addition assay (18). Briefly, a strain carrying an ectopic copy of *ADE2* adjacent to a stretch of $TG_{1\text{-}3}$ repeats and an HO endonuclease site is arrested at G2/M with nocodazole, a time when telomere addition has been shown to be active. The HO endonuclease is induced in the strain by incubation in galactose. At various times after induction, samples are taken; DNA is prepared and digested with SpeI, generating the fragments indicated. These fragments are separated by gel electrophoresis and blotted and probed with an *ADE2* probe. Note that the strain carries an additional copy of *ADE2* at its endogenous chromosomal location not shown in the diagram. See Materials and Methods for details. (B) Hybridization of *ADE2* probe to SpeI-digested DNA samples taken at the indicated times after induction of HO endonuclease by the addition of galactose. PRE-Parental DNA uncut by HO; LM, HO-cut DNA; SM, telomeric addition; INT, SpeI-fragment containing the *ADE2* gene at its endogenous chromosomal location as internal hybridization control. (C) Quantitation of telomere addition onto $TG_{1\text{-}3}$/HO ends after normalization of SM to the INT fragment. (D) Quantitation of disappearance of the LM band after normalization of LM to the INT fragment. The Y-axis in panels C) and D) represents normalized phosphorimager readings in arbitrary units.

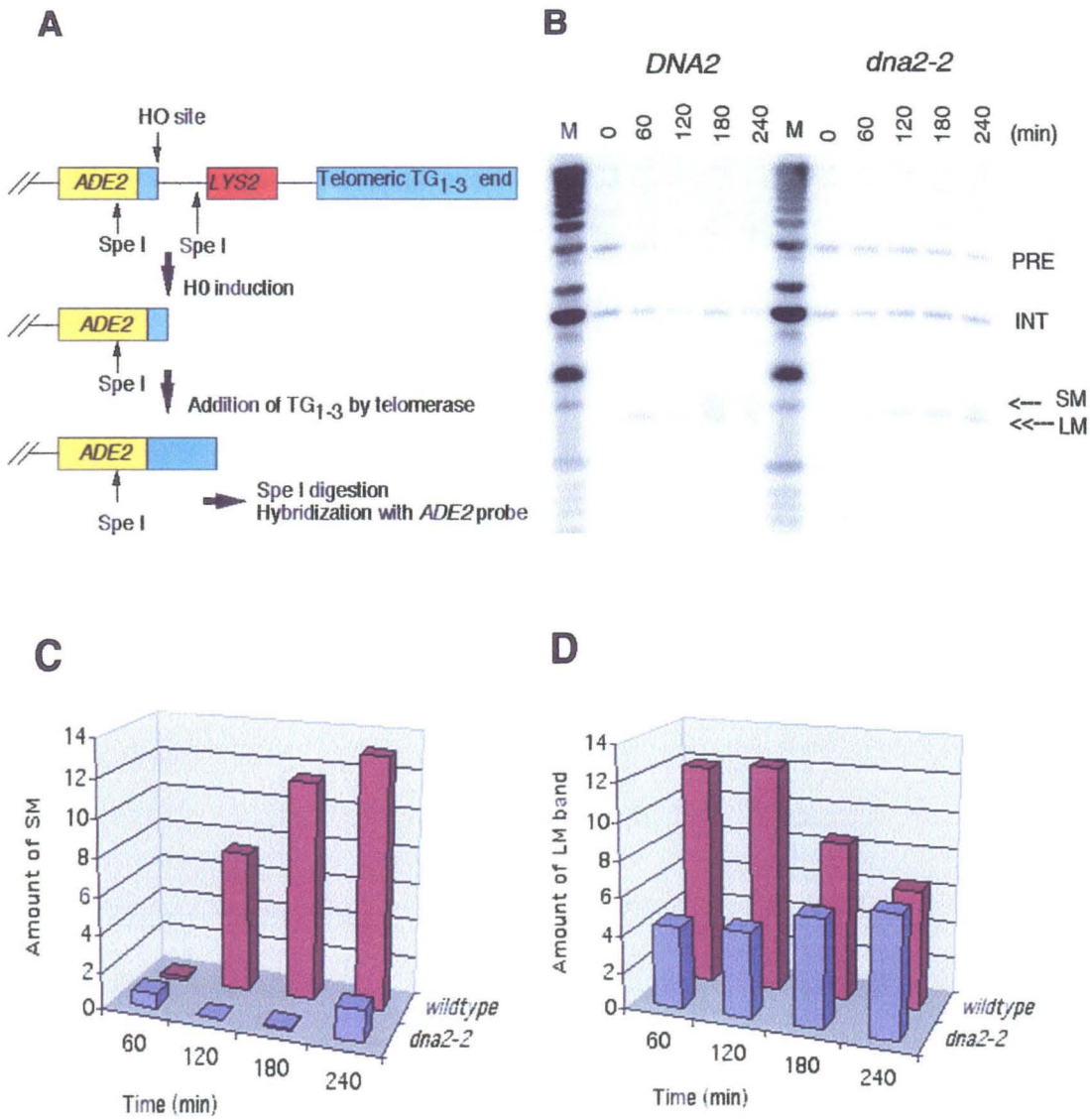

A
HO site
ADE2
LYS2
Telomeric TG1-3 end
Spe I
Spe I
HO induction
ADE2
Spe I
Addition of TG1-3 by telomerase
ADE2
Spe I
Spe I digestion
Hybridization with ADE2 probe
B
DNA2
dna2-2
M 0 60 120 180 240 M 0 60 120 180 240 (min)
PRE
INT
<--- SM
<<--- LM
C
Amount of SM
14
12
10
8
6
4
2
0
60
120
180
240
Time (min)
wildtype
dna2-2
D
Amount of LM band
14
12
10
8
6
4
2
0
60
120
180
240
Time (min)
wildtype
dna2-2

Figure 6

Figure 7. Telomerase-deficient mutant deficient in *dna2* shows accelerated senescence. (A) Increased senescence in *dna2-2est2Δ* mutants. A diploid strain est2dna2-2 (MATα *est2::KanMX4 leu2Δ met15Δ ura3Δ/MATa dna2-2::LEU2 KANMX4 leu2Δ met15Δ ura3Δ*) heterozygous at both the *EST2* and *DNA2* loci was sporulated and dissected. After 2 days (about 25 generations), the spores were replica plated onto G418-containing medium lacking leucine. Individual spores from a tetrad with the tetratype genotype were then restreaked on rich medium and grown to single colonies (about 25 divisions). *DNA2$^+$EST2$^+$*, *dna2-2est2*, *DNA2$^+$est2Δ* and *dna2-2EST2$^+$* were restreaked on a YPD plate. The same experiment was performed with strain *dna2-2est1Δ* heterozygous diploid (not shown, but see Fig. 7B).

(B) Telomere maintenance in senescing *estΔ* and *dna2est1Δ* mutants. A diploid strain (MATα *est1::KanMX4 leu2Δ met15Δ ura3Δ/MRTa dna2-2::LEU2 KANMX4 leu2Δ met15Δ ura3Δ)* heterozygous at both the *EST1* and *DNA2* loci was sporulated, dissected and the genotype of the spore products determined, as in Fig. 7A. A tetrad with the tetratype genoytpe was then streaked onto a YPD plate at 30°C as in Fig. 7A. From this plate, *est1Δ* and *dna2-2est1Δ* colonies were inoculated into 5 ml of YPD medium. After the cultures grew to saturation, 2.5 μl of each culture was added to 5 ml of fresh YPD medium. The inoculations were repeated six times. The type II survivors appeared in the *dna2-2est1Δ* strain after two inoculations and in the *est1Δ* strain after four inoculations. Telomere maintenance was assessed by preparation of DNA from each culture, digestion

with XhoI, gel electrophoresis, blotting and hybridization of the telomere blot with a $C_{1-3}A$ telomere probe as described (39). Lane 1, marker, large spot in the Y' telomere bracketed region is about 1 kb; lane 2, *DNA2*$^{+}$; asynchronous culture; lane 3, *dna2-2*, asynchronous culture; lane 4, *est1Δ* after one passage; lane 5, *dna2-2 est1Δ* after one passage; lane 6, *est1Δ* after two passages; lane 7, *dna2-2 est1Δ* after two passages; lane 8, *est1Δ* after three passages; lane 9, *dna2-2 est1Δ* after three passages; lane 10, *est1Δ* after four passages; lane 11, *dna2-2 est1Δ* after four passages; lane 12, *est1Δ* after five passages; lane 13, *dna2-2 est1Δ* after five passages; lane 14, *est1Δ* after six passages; lane 15, *dna2-2 est1Δ* after six passages. The smear beginning above the bracket denoting Y' telomeres and the larger rearranged bands represent the putative Type II intermediates. These experiments were performed before publication of a higher resolution assay for Type I and type II (58, 59).

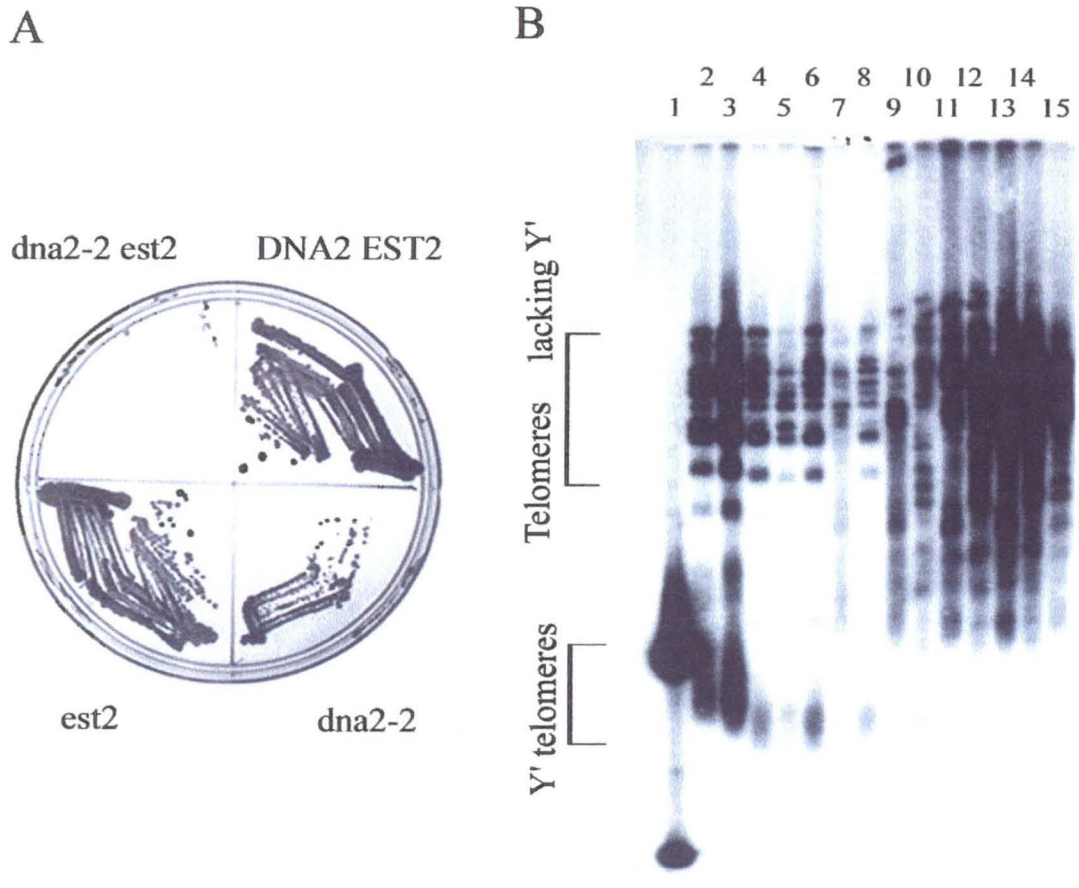

A
dna2-2 est2
DNA2 EST2
est2
dna2-2
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Telomeres lacking Y'
Y' telomeres

Figure 7

Molecular and Cellular Biology **(in press)**

DNA REPLICATION AND YEAST LONGEVITY

Laura L. Mays Hoopes[1], Martin Budd, **Wonchae Choe**, and Judith L. Campbell

Braun Laboratories 147-75
California Institute of Technology
Pasadena, CA 91125
Telephone: 626-395-605 FAX: 626-405-9452
E-mail: jcampbel@cco.caltech.edu

[1]Department of Biology and Molecular Biology Program
Pomona College, Seaver South Laboratory
Claremont, CA 91711

Running title: Replication Stress Reduces Normal Yeast Life Span

Key words: Dna2 helicase/nuclease, recombination, aging, Werner syndrome

Corresponding Author: Judith L. Campbell

Yeast has been used extensively as a model organism for identifying genes potentially involved in mammalian replicative senescence. However, the contribution of defects in DNA replication to determination of yeast longevity has never been directly investigated. We show that a yeast helicase/nuclease, *DNA2*, which is essential for DNA replication, is required for normal life span. Life span reduction reflects normal yeast aging since it is accompanied by age-related transcriptional silencing defects and genomic instability. Shortened life span is not limited to *dna2* mutants, since several other replication mutants also show premature aging. Based on these results, and on other recent results suggesting that replication may not be as processive as previously thought, we propose that replicative failure due to spontaneous, endogenous DNA damage may underlie some forms of mammalian replicative senescence. This may imply that the genomic instability, segmental premature aging symptoms, and cancer predisposition associated with the human helicase diseases such as Werner, Bloom, and Rothmund-Thompson syndrome are also related to replicative stress.

Saccharomyces cerevisiae has recently been shown to be a promising model system for the identification of genetic pathways involved in determining longevity in more complex organisms. In yeast, there is a spiral form of aging, in which mother cells in each division increase in age by one generation while each new bud is produced at age zero (Mortimer and Johnston 1959; Jazwinski 1990). The total life span of yeast is counted as the number of times the mother cell is able to bud. This form of aging resembles mammalian cell replicative senescence (Hayflick 1965), the finite number of divisions of a cell in culture, with two differences. Yeast cell division is asymmetric rather than symmetric with respect to age, and yeast cells lyse at the end of the life span rather than undergoing some form of differentiation.

Yeast cells at the end of their life span exhibit a set of characteristic phenotypes that serve as markers for aging-related phenomena. These phenotypes include increased cell size, increased time per cell division (Jazwinski 1990; Kim et al. 1999b), sterility due to deregulation of transcriptional silencing (Smeal et al. 1996), nucleolar enlargement and fragmentation (Sinclair et al. 1997), amplification of rDNA and formation of Extrachromosomal rDNA Circles (ERCs) (Sinclair and Guarente 1997), and altered UV resistance (Kale and Jazwinski 1996). The analysis of yeast mutants that have shortened life spans, such as *sgs1*, a homolog of WRN and BLM helicases, suggested that nucleolar fragmentation and production of rDNA circles occurs early in these mutants (Sinclair and Guarente 1997). The helicase encoded by *SGS1* is thought to be involved in the down-regulation of homologous recombination in the rDNA repeats, and based on this and other findings it has been proposed that a reasonable model for yeast aging is that hyper-recombination in the rDNA produces ERCs that accumulate preferentially in mother cells

and ultimately lead to cell death (Sinclair and Guarente 1997; Sinclair et al. 1997; Sinclair et al. 1998). Paradoxically, however, genes that eliminate homologous recombination (*RAD50*, *RAD51*, *RAD52*, and *RAD57*) lead to shortened life span and do so without giving rise to ERCs (Park et al. 1999). Furthermore, in cells recovering from stationary phase arrest, life span is also shortened without the appearance of ERCs (Ashrafi et al. 1999); and, conversely, in certain petite strains, life span is extended but there is an increase in ERCs (Kirchman et al. 1999). Finally, it has recently been discovered that wildtype cells and *sgs1* mutants that have transited the same number of cell divisions accumulate equal numbers of ERCs, but that *sgs1* mutants appear to be more sensitive to their presence (Heo et al. 1999; McVey et al. 2001). Thus, ERCs are not the sole aging factor in yeast, though they are important (Guarente and Kenyon 2000). Also, in organisms other than yeast, accumulation of rDNA copies does not correlate with replicative senescence (Peterson et al. 1984). Our studies were designed to test whether the rDNA phenotype in yeast could be secondary to some systematic DNA damage that increases recombination frequency to accomplish repair.

Two types of mutations could lead to increased recombination: 1) mutations that cause an increase in the basal level of recombinogenic damage or 2) mutations that are defective in the processing of such damage and that result in accumulation of intermediates that are lethal. The type of damage that leads to short life span seems to be quite specific, since unlike deletions of genes affecting the recombination pathway, deletions of *RAD1*, affecting the nucleotide excision repair pathway and single-strand annealing repair, *RAD7*, affecting the nucleotide excision repair pathway, and *RAD26*,

affecting the transcription coupled repair pathway, have normal life spans (Park et al. 1999).

Several observations led us to test the hypothesis that DNA damage during DNA replication, arising, for example, from slow moving, stalled, or collapsed replication forks or intermediates in their repair, might be a factor contributing to life span reduction. Recent studies in bacteria suggest that DNA replication is more likely to be interrupted than previously supposed (Kogoma 1997; Kuzminov 1999 for reviews). Replication forks assembled at origins encounter blocks, for instance in the form of endogenous DNA damage, secondary structures, protein complexes involved in other pathways. If the damage is a template strand interruption, fork encounter may result directly in a double-strand break. Forks encountering other blocks may be converted to four-way junctions by branch migration and reannealing of the newly synthesized strands. Processing of this four-way junction in *Escherichia coli* by RuvABC may result in the generation of a recombinogenic double-strand break (DSB), while RecBCD processing leads to recombination-induced replication restart and removal of damage (Seigneur et al. 1998). Alternatively, paused forks may simply lead to disassembly of the replisome followed by replication restart (Kuzminov 1999). In bacteria, there is good evidence that replication mutants affecting helicases give rise to such damage, and that recombination is involved in the rescue of the replication forks (Michel et al. 1997; Seigneur et al. 1998). Failure of timely rescue leads to genetic instability. Replication fork demise may also occur in eukaryotes and be recombinogenic, since recombination intermediates increase spontaneously, during DNA replication in yeast in the absence of any exogenous DNA damaging agent. Also, specific replication mutants increase the frequency of replication-

related recombination, especially at the non-permissive temperature (Hartwell and Smith 1985; Zou and Rothstein 1997). In addition, several DNA replication mutants accumulate double-strand breaks (DSBs) and require *RAD52*, essential for recombination or DSB repair, for viability, suggesting that recombination is required to repair the DSBs arising from replicational stress (Holmes and Haber 1999). Finally, early studies in higher eukaryotes showed that DNA damage is converted to DSBs and stimulates sister chromatid exchange only if damaged chromosomes are allowed to traverse S phase (Wolff et al. 1974; Wang and Smith 1986).

We first studied a known DNA replication mutant, *dna2*. Dna2p is a yeast helicase/nuclease (Budd and Campbell 1995; Budd et al. 1995; Budd and Campbell 1997; Bae et al. 1998). The helicase domain of *DNA2* is closely related to WRN at the primary sequence level, though not as closely as is *SGS1*. What is striking, however, is that Dna2p, like WRN, contains both helicase and nuclease activities in the same polypeptide (Shen and Loeb 2000). *DNA2* is essential for viability (Kuo et al. 1983; Budd and Campbell 1995; Budd et al. 1995). Dna2p is thought to be an intrinsic component of the replication apparatus and to either compensate for or cooperate with the *RAD27*-encoded FEN-1 in maturation of Okazaki fragments (Budd and Campbell 1995; Budd and Campbell 1997). Important in our choice of *DNA2* as a test gene, inactivation of Dna2p leads to fragmentation of replicating chromosomal DNA (Budd and Campbell 1995; Kang et al. 2000). Furthermore, *dna2* mutants are defective in repair of DSBs caused by X-rays or bleomycin, agents that mimic oxidative damage, or by MMS (Formosa and Nitiss 1999; Budd and Campbell 2000). Strains carrying *dna2* mutations require *RAD52* (essential for homologous recombination, DSB repair) for optimal growth suggesting that

DNA damage resulting from the failure of Dna2 to perform its function requires recombinational repair (Budd and Campbell 1997; Budd and Campbell 2000). Mitotic recombination and chromosome loss are not elevated in most *dna2* mutants, though chromosome loss may be elevated in *dna2-22* (Hartwell and Smith 1985; Fiorentino and Crabtree 1997; Formosa and Nitiss 1999). *DNA2* belongs to the epistasis group with *RAD6* and *RAD18*, which are involved in the postreplication repair pathway (Budd and Campbell 2000). The role of Dna2 in postreplication repair could be the same as its role in DNA replication per se, since postreplication repair may involve new DNA synthesis from sister templates (Prakash 1981; McDonald et al. 1997; Budd and Campbell 2000). Finally, overexpression of the N-terminus of *DNA2* reduces telomere position effect, the silencing of genes in telomere proximal positions, suggesting a role in silencing, a major aging pathway (Singer et al. 1998).

As shown in Fig. 1A, *dna2* mutants do have severely shortened life spans. We observed a variety of mutants, including the temperature sensitive *dna2-1* at a permissive temperature, *dna2-2*, which is not temperature sensitive and has only a slightly reduced doubling time compared to wildtype (Formosa and Nitiss 1999), and *dna2-20* and *dna2-21*, which require osmotic support (Fiorentino and Crabtree 1997). None of these mutants shows elevated mitotic recombination or chromosome loss (Fiorentino and Crabtree, 1997; Formosa and Nitiss, 1999). The average life span for *dna2-1* was 8.1 ± 2.5 generations, that for *dna2-2* was 8.2 ±2.5 generations, that for *dna2-20* was 4.9 ± 1.9 and that for *dna2-21* was 7.2 ±2.6 generations. Thus, all four mutants showed substantially shorter average life spans than their wild type parent strains (30.4 ± 5.4 generations for the parent of *dna2-1* and 26.55±13.91 generations for the parent of *dna2-*

2). All *dna2* strains differed significantly from the wildtype at p=<0.001. *dna2* mother cells near the end of their life span became enlarged (Fig. 2A) and showed increase in cell cycle length (not shown). Daughters of old mother cells returned to normal cell size and cell cycle length within one or two cell divisions, indicating that, as in wildtype, aging is asymmetric. Mutant *dna2* mothers also became sterile as they progressed through their life span (Table 1) demonstrating a disruption of transcriptional silencing, another age-specific phenotype (Smeal et al. 1996). Only one difference from wildtype aging was noted. *dna2* cells ceased dividing as budded cells (>90%), whereas wildtype mothers stop growing as largely unbudded cells (60% unbudded, 20% small buds, and 20% large buds). The large budded phenotype would be expected for a DNA replication mutant. We conclude that Dna2p is required for maximum life span in yeast. Since a regulatory effect on aging is more convincing if life span can be extended, we studied overproduction of Dna2p. Overproduction of Dna2p, however, has little or no effect on life span (Fig. 1B). In fact, overproduction leads to a small life span reduction, as might be expected since overproduction of Dna2p can result in lethality (Parenteau and Wellinger 1999).

Since *SGS1* and *DNA2* both encode helicases, it was of interest to test their relationship in life span determination. We investigated whether an *sgs1Δdna2* double mutant had a more severe defect in life span than either single mutant alone. We found that *sgs1Δdna2-2* double mutants were synthetically lethal in the genetic background that we chose but had the same doubling time as single mutants when osmotically supported with 0.5 M sorbitol, similarly to *dna2-20* strains (Fiorentino and Crabtree 1997). Thus, life spans of the double mutants were determined in medium containing sorbitol. Fig. 1C

shows that the life span of the double mutant *dna2-2sgs1*Δ differed significantly (p = 0.03 by Mann Whitney test; p = 0.02 by t test) from that of *dna2-2*, and even more so from *sgs1Δ*. To insure this observation was also true in genetic backgrounds where the two mutations were not synthetically lethal, we also measured life span in double mutants that were viable without addition of sorbitol (Budd and Campbell 2000). The life span of the *dna2-2* mutant is 8.2 ± 2.5 generations and that of the *sgs1Δ* mutant is 12.6 ± 6.3 generations in this background. The double mutant again had a significantly shorter (p=0.03) average life span than either single mutant, 6.5 ± 2.9 of generations. Thus, the aging phenotype of the double mutants is not dependent on genetic background. Taken together with differences in phenotype of the single mutants documented previously, the fact that *dna2* and *sgs1Δ* mutations clearly act additively leads us to propose that the two helicases have different substrates, accounting for their independent contributions.

We next tested for additional phenotypes documented as characteristic of late generation wildtype cells to further insure that we were observing accelerated aging (Fig. 2A-C). For this, we used the repeated rDNA locus as an assay. We first asked if *dna2-2* mutants showed age-related nucleolar enlargement and fragmentation. In wildtype mother cells, the nucleolus is unitary and resembles a cap on the nucleus early in life, but becomes enlarged and fragmented at around the average life span (Sinclair et al. 1997). Fig. 2A shows *dna2-2* cells stained with DAPI to reveal the entire nucleus (dark blue) and with monoclonal antibody to the abundant nucleolar protein Nop1p (bright green), a protein involved in ribosome biogenesis (Aris and Blobel 1988). The young cells all have typical nucleolar morphologies (Fig. 2A, Young). Greater than 90% of the *dna2-2* cells isolated at seven to nine generations, about half their maximum life span, however, have both

enlarged and/or fragmented nucleoli (pale/green or aqua Nop1staining) (Fig. *2A,* Old). The change in Nop-1 color between young and old cells is probably due to the large amount of ribosomal DNA (see below) and/or relative dilution of Nop-1. The striking difference between the populations of young and old cells confirms that the nucleolar phenotype is not a feature of the general *dna2* mutant population but, as in wildtype, that it is directly correlated with age.

Wild-type mother cells accumulate rDNA, both intrachromosomally and in the form of extrachromosomal circles (ERCs), as they progress through their life span (Sinclair and Guarente 1997). We therefore compared the rDNA in young *dna2-2* cells and in cells at about half their maximum life span. As shown in Fig. 2B, equal amounts of undigested DNA from the young and old cells were separated on an agarose gel by conventional electrophoresis (Fig. 2B, left) and analyzed by Southern blotting with an rDNA probe (Fig. 2B, right). When the gel was blotted and probed with labeled rDNA, the amount of rDNA-specific hybridization increased dramatically in the old versus young cells. In addition, there seemed to be significant rDNA-specific degradation compared to young cells. This degradation was not noted on the EtdBr stained gel and thus did not represent general DNA degradation or a problem with the method used to prepare DNA. Nor was it observed in DNA isolated from eight-generation wildtype cells (not shown). Identification of ERCs (5 kb) by 1D gel analysis was hampered by the extensive degradation of the rDNA in the *dna2-2* mutant. Circular DNA can be separated from linear degradation products by 2D gel analysis in the presence of intercalating agents (Fig. 2C)(Sinclair and Guarente 1997). As a positive control for ERCs we used the isogenic *sgs1Δ* strain, which accumulates ERCs early in its lifespan (Sinclair and

Guarente 1997). ERCs are visible as the dark arc of topoisomers appearing below the diagonal of linear DNA in the *sgs1Δ* DNA in Fig. 2C. In the *dna2-2* strain at about eight generations, Fig. 2C, left, ERCs are observed as a faint arc of much lower intensity than that in the *sgs1Δ* strain. These results were repeated in at least three separate experiments and eight-generation wildtype cells showed no ERCs under the conditions of our experiments (not shown). We conclude that, as in old wildtype or *sgs1* mutants, late generation *dna2* mutants also show genomic instability in the rDNA (Fig. 2B). As shown in Fig. 2C, this is reflected partially but not exclusively in ERCs, which may fail to replicate efficiently in the *dna2* mutant. Thus, while accumulation of ERCs is important for the aging of *sgs1Δ* (Sinclair and Guarente 1997), high copy numbers of ERCs probably do not account for the cessation of cell division in old *dna2* mothers. Since *DNA2* is a replication gene, we propose that the increased rDNA in old *dna2* mutants is a symptom of an underlying defect in DNA replication leading to recombinogenic structures in the rDNA. The difference between young and old *dna2* populations shows that this is specific to aging. The observed increase in fragmentation of the rDNA with increasing age is evidence of DSB formation, as has been observed in *dna2* mutants at the restrictive temperature (Kang et al. 2000). While DNA replication mutants have been shown to be hyper-recombinogenic by other assays (Hartwell and Smith 1985), this phenotype has not been related to cellular aging in any previous study.

If replication-related damage is involved in the events occurring in the rDNA during aging in the *dna2* mutants, then *DNA2* should be found in the nucleolus during S phase and should be associated with replicating rDNA. While *DNA2* is essential for completion of DNA replication, the bulk of the DNA is replicated in *dna2* mutants. Thus,

it has not been formally shown that Dna2p replicates the rDNA. Furthermore, Dna2p has never been shown to be directly associated with any replicating DNA. Chromatin crosslinking immunoprecipitation (ChIP) studies were carried out as a function of cell cycle phase position in order to assess whether the Dna2p was associated with rDNA and whether it was associated during DNA replication (Fig. 2D). Cells were synchronized with mating pheromone before Start (early G1). They were then released into a synchronous cell cycle and the association of *DNA2* with two different regions within the rDNA was assessed. FACS analysis demonstrated that cells entered S phase about 40 minutes after release from pheromone and that most of the cells contained a G2 DNA content by 75 min (not shown). As shown in Fig. 2D, Dna2p is absent from the rDNA during G1, arrives at the rDNA at the beginning of S phase, associates with the rDNA maximally in S phase, and shows reduced association in G2 phase. Probes from two different rDNA regions, one spanning the Replication Fork Barrier (RFB, a site where replication forks are programmed to pause) and one within the 35S-coding region showed similar timing of association (Fig. 2D, and see below). The same dramatic difference between G1 and S/G2 was seen for many different numbers of cycles of amplification (data not shown), suggesting the robustness of the result. The temporal pattern of association of *DNA2* with the rDNA suggests that its primary role in the nucleolus is associated with the replication of the rDNA (S phase) or with repair of rDNA damaged during replication or of damage remaining after passage of the replication fork (S and G2 phases, but not G1). The parsimonious interpretation is that the rDNA amplification in the *dna2* mutant arises due to replicative stress, resulting in an increase in recombination in an attempt to repair replication fork damage.

It is important to emphasize that we are not proposing that the role of *DNA2*, which plays a role in global DNA replication, is limited to the rDNA. Rather, the effects on this repeated sequence provide an assay (breakage and amplification) for similar types of DNA damage and repair that occur due to failure(s) of *dna2* during DNA replication or repair of replication related damage at single-copy sequences throughout the genome (Budd and Campbell 1995; Kang et al. 2000), which might be detected with different assays (Chen et al. 1998). Furthermore, Zou and Rothstein (1997) have used the rDNA as an assay to demonstrate directly that Holliday recombination intermediates accumulate in the rDNA, in every cell cycle, appearing maximally during S phase, i.e., with the same temporal pattern as Dna2p association with rDNA. They also showed that at least six DNA replication mutants, including *cdc9*, which is synthetically lethal with *dna2* (Ireland et al. 2000), increase the frequency of Holliday structures in the rDNA. They propose, as we do for *dna2*, that spontaneous damage during DNA replication gives rise to these structures throughout the genome. What is new here is that we find that this genetic instability is correlated in *dna2* mutants with shortened life span.

If the defect that underlies the genomic instability observed in the yeast rDNA reflects global replication stress, and if this stress contributes to premature aging, then other DNA replication mutants might be expected to exhibit reduced life spans. In Fig. 3, life spans of cells with mutations affecting pol α, FEN-1, and CTF4, *pol 1-14* and *pol 1-17*, *rad27Δ*, and *ctf4Δ*, respectively, are shown. All three of these genes have been shown to interact genetically with *dna2* (Budd and Campbell 1997; Formosa and Nitiss 1999). Pol α is essential for initiation of Okazaki fragments; Fen1 is involved in maturation of Okazaki fragments; and *CTF4* prevents chromosome loss (Kouprina et al. 1992) and

encodes a protein that binds to the catalytic subunit of pol α, Poll p (Miles and Formosa 1992a; Miles and Formosa 1992b). As anticipated, three of the four mutations tested that affected DNA replication also reduced life span (*rad27Δ,* average life span 11.6 ± 7.0 generations, *ctf4Δ*, average life span 6.4 ± 4.4 generations, *pol 1-14*, average life span 11.5 ± 8.6 generations). One DNA polymerase mutant, *poll-17*, had an average life span comparable to its isogenic parent (21.2 ± 7.6 generations) at the permissive temperature. Interestingly, this mutant has a normal growth profile at the permissive temperature and serves as an important comparison for *poll-14*, which shows serious growth defects and abnormally high DNA content by FACS analysis at both permissive and restrictive temperatures (Budd et al. 1989). Extensive nucleolar fragmentation and enlargement occurs in the aged *poll-14* (data not shown) as in *dna2-2* (Fig. 2A). These results support the idea that a replication defect, either in fork propagation or in repair of damaged replicating DNA, shortens life span.

We are implying that the premature aging of DNA replication mutants represents an exaggerated case of spontaneous replication errors that occur in wildtype cells in every generation and that eventually lead to yeast aging. In the replication mutants, it is not clear whether the basal level of damage is increased or if the sensitivity to the damage is increased during S phase, for instance due to failure to repair damage. Either scenario might have the same outcome. This replication lesion model for yeast aging, and thus replicative senescence, is not inconsistent with two specific hypotheses for causes of yeast aging based on previous work, but adds an additional dimension, and suggests how the pathways may intersect. First, the *recombinational aging model* specifically hypothesized that aging results from inappropriate recombination in the rDNA repeats,

resulting in extrachromosomal rDNA circles, which are asymmetrically inherited. This hypothesis was originally supported by the ability of ectopic generation of an ERC to accelerate aging (Sinclair and Guarente 1997). Recently enough cases have been documented in which there is either no correlation or an anti-correlation between aging and ERCs that ERCs no longer provide an explanation of the mechanism of yeast aging; instead they appear to be another symptom and the model has been modified (Sinclair and Guarente 1997; McVey et al. 2001). The replication model we suggest simply suggests that recombination is a sequel to a more primary defect in replication fork propagation/postreplication repair of replication lesions. It nicely accommodates premature aging defects in recombination mutants like *rad52* (Park et al. 1999), because recombinational repair may be necessary for repair of replicative damage. It is supported by the observed "spontaneous" increase in recombination specifically during S phase and in DNA replication mutants (Zou and Rothstein 1997). Indeed, there is beginning to be a wide perception that recombination is a repair system for DNA damage brought about by or during DNA replication (Kuzminov 1999).

Second, a *defective silencing model* has been proposed in which aging results from a loss of the ability to silence inappropriate gene expression (Kennedy et al. 1997; Imai et al. 2000; Lin et al. 2000). This mechanism is supported by life span extension from deleting a histone deacetylase gene that is required for silencing at some loci (Kim et al. 1999a), age-related losses in telomeric gene silencing (Kennedy et al. 1997), relocation of the Sir proteins to the rDNA during aging (Kennedy et al. 1997), life span reduction in a *sir2* deletion mutant, and life span extension by overproduction of *SIR2* (Kaeberlein et al. 1999). In the replication stress model of aging, the relocation of the Sir

proteins noted during aging could be directed to aid in remodeling of chromatin during repair of damage to the replication fork. rDNA recombination is increased in *sir2* mutants, and the link between silencing, recombination and replication could occur at this level (Gottlieb and Esposito 1989). An increasing number of observations implicate replication genes in silencing (Miller and Nasmyth 1984; Axelrod and Rine 1991; Ehrenhofer-Murray et al. 1999; Martin et al. 2000; Zhang et al. 2000). In addition, overexpression of Dna2p leads to derepression of genes inserted at telomeres and the silent mating type loci (Singer et al. 1998). Also, old *dna2* cells show early sterility (Table 1) suggesting defects in silencing in the silent mating type loci and reorganization of the Sir complex. During normal aging, a likely source of replication errors is endogenous oxidative DNA damage, which is likely to increase at high metabolic rates. The recent demonstration that Sir2 histone deacetylase requires NAD^+ as a cofactor links the extension of life span by overproduction of *SIR2* to the metabolic state of the cells, the greater the metabolic rate, the more *SIR2* is needed (Imai et al. 2000; Lin et al. 2000). Therefore, reformation of chromatin after recombinational repair of replication blocks due to oxidative damage in rapidly growing cells might require *SIR2*. We have found that introduction of an extra copy of *SIR2* into *dna2* mutants increases the maximum lifespan significantly (Budd and Campbell, unpublished) which would be consistent with the latter proposal.

The premature aging of *sgs1* is consistent with the replicative damage hypothesis we propose. The double mutant *sgs1Δsrs2Δ* is inviable, and *sgs1$_{ts}$srs2Δ* strains are defective in DNA synthesis and rDNA transcription, suggesting *SGS1* may be at the replication fork (Lee et al. 1999). This lethality can be overcome by a *rad51* mutation

(Gangloff et al. 2000; McVey et al. 2001). One way to explain the suppression is that a putative intermediate in damaged replication fork processing accumulates in the double mutant but is not lethal if it is prevented from entering the recombination pathway (Kogoma 1997). Others have found that Sgs1p is an integral component of the S-phase checkpoint response in yeast, binding to Rad53p and in the same epistasis group with pol ε (Frei and Gasser 2000). They suggest the role of Sgs1p is to monitor replication fork progress, for instance, to detect stalled forks. This suggestion would make the results with *sgs1* consistent with the replication stress hypothesis for yeast life span determination. The increased severity of the defect in *sgs1dna2* double mutants suggests divergence of function between the two helicases at some point in the complicated process. One possible scenario is that Sgs1p is required to resolve fork damage (Karow et al. 2000), while Dna2p is required to prevent damage.

These results may shed light on human helicase diseases, which may also be diseases of DNA replication. Recently, BLM helicase has been proposed to be an antirecombinase, which like bacterial RuvA,B can promote branch migration of Holliday structures and thus might resolve reversed replication forks without entry into the recombination pathway (Karow et al. 2000). WRN, on the other hand, encodes a helicase/nuclease, similar to Dna2p in that the helicase and nuclease seem to act in concert biochemically, though perhaps differing in substrate specificity (Shen and Loeb 2000). WRN helicase interacts with replication proteins, both BLM and WRN cell lines show defects consistent with defects in DNA replication, and the BLM$^-$/BLM$^-$ knockout is an embryonic lethal (see review in Yamagat et al., 1998). It will be interesting to test

complementation of yeast *dna2* mutant phenotypes by vertebrate WRN and BLM genes, as has already been done for yeast *sgs1Δ* strains (Yamagata et al. 1998; Heo et al. 1999).

In conclusion, since aging is likely due to multiple factors, we mention that enhanced response to stress is implicated in lengthening life span. In yeast, overproduction of Lag1p or Ras2p leads to an extension of the life span, possibly by controlling anti-stress mechanisms (Jazwinski 1999). These mechanisms are not addressed in the current study, but it may be that they interact with the mechanisms we have discussed in that they may lead to replicative damage.

Material and methods

Life span determination

Life spans of virgin mothers were determined by dissection as described (Jazwinski 1990). Strain *dna2-1* was grown at 23°C and all other mutants were at 30°C. Statistical significance of differences in life spans was determined using a t test and confirmed using a Mann Whitney non-parametric test using StatMost software (Dataxiom Software Inc., Los Angeles, CA).

Nucleolar morphology in aging yeast cells

Cultures of *dna2-2* cells were labeled with biotin, grown, and then recovered after 8-9 generations using streptavidin paramagnetic beads as described by others (Sinclair et al. 1997). Sulfo NHS LC biotin was from Pierce. Paramagnetic streptavidin coated beads were from PerSeptive Biosystems (Framingham, MA) and were used at 5 mg/ml. An aliquot of the cells isolated were stained for 20 minutes in 1 μg/ml calcofluor (Sigma Chemical Company, St. Louis, MO, Fluorescent Brightener 28), washed in phosphate buffered saline and the average bud scars determined for 15 to 20 cells in UV epifluorescence, verifying the age of the cells.

Immunofluorescence was performed by a slight modification of the method of Pringle (Pringle et al. 1991). Cells were fixed in formaldehyde, digested with Zymolyase (ICN) for 5 to 30 minutes until phase dark, washed, and attached to polylysine-coated slides. Slides were blocked for an hour, stained with primary antibody for an hour, rinsed five times, and stained with secondary antibody for an hour and rinsed five times. The primary antibody was A66 mouse monoclonal antibody to Nop1p or yeast fibrillarin, an

abundant nucleolar protein (Aris and Blobel 1988). Donkey FITC- labeled anti-mouse secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Nuclei were stained for 5 seconds with 0.1 μg/ml DAPI (4',6-Di Amidino-2-Phenyl Indole, Sigma, St Louis, MO) and rinsed for 5 seconds with deionized water. Cells were mounted in Vectashield (Vector Laboratory, Burlingame, CA). Stained cells were photographed with a Hamatu Digital Camera using a Nikon Eclipse TE300 inverted microscope and Metaphore imaging software. All images are at the same magnification.

Analysis of rDNA in young and old cells

Young and old cells were prepared by the biotin method as above. DNA was isolated by gentle spheroplasting, and methods for 1D analysis to separate circular rDNA from total genomic rDNA (Fig. 2B) and for 2D gel electrophoresis in the presence of intercalators were similar to those described in (Sinclair and Guarente 1997). Chloroquine gels were run in 1% (w/v) Tris-acetate-EDTA agarose at 1.3 V/cm for 39 h in 0.6 μg/ml chloroquine in the first dimension and 20 h in 3 μg/ml chloroquine in the second dimension.

Chromatin immunoprecipitation assays

Cells were synchronized with mating pheromone and released as described (Dua et al. 1998). Samples were taken at the indicated times and ChIP assays performed as described by Tanaka and Nasmyth (Tanaka et al. 1997). *DNA2* was tagged with 11 myc epitopes and integrated at the *DNA2* locus in strain W3110*pep4bar1*(RAD5^{+}). Anti-myc monoclonal antibody 9E10 was used for immunoprecipitation. One set of PCR primers is

from the 35S RNA transcription region as indicated in the diagram in Fig. 2D and one spans the RFB (Kobayashi et al. 1992). The RFB site was chosen as the starting point for designing the probe in the *Saccharomyces* Data Base (SDB, Stanford) and was assigned the nucleotide number 5000. The RFB probe spanned nucleotides 4971-5320. PCR reactions were carried out for different numbers of cycles to insure that results were in the linear range as described (Tanaka and Nasmyth 1998).

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Table 1. *Sterility test for dna2-1MATa, W303MATa, and sgs1ΔMATa.*

The ability of a *dna2-1MATa* strain to respond to 10 μg/ml alpha factor very early in its life and at near the halfway point of its life span, which is 8 generations, was examined. The ability of *dna2-1MATa, sgs1ΔMATa,* and W303*MATa* strains to respond to alpha factor was examined as described (Smeal et al. 1996) using sterile filter paper rectangles soaked with 10 μg/ml alpha factor. Responses were recorded as the number of cells with schmoo morphology after 3 hours and 5 hours of exposure. After removal of the filter paper, cells were moved to 0.5 cm beyond the site of the test and examined to make sure they recovered and resumed division. Cells that failed to resume division were not counted in the data set on the assumption that they could be on the road to death and thus beyond possibility of response.

Generations	Schmoos	Total	Percent sterile
W303[a]			
2-3	34	44	16%
7-9	20	20	0%
9-15	13	24	46%
dna2-1			
2-3	43	50	14%
2-3	10	20	50%
5-8	2	13	85%
5-9	2	13	85%
sgs1Δ			
6-10	4	10	60%
13-19	1	12	92%

Figure Legends

Figure 1A. *dna2* mutants have reduced life span. Average life spans (number dissected, N, given in parentheses) of *dna2* mutants: *dna2-1*, 8.1± 2.5 generations (N=25); *dna2-2*, 8.2±2.5 generation (N=25); *dna2-20*, 4.9±1.9 generations (N=36). The *DNA2*$^+$ curve shown is strain SS111 (wildtype parent of *dna2-1*), 30.4±5.4 generations (N=37). The isogenic wild type strain for *dna2-20* is not available (pers. comm.) (Fiorentino and Crabtree 1997). The isogenic parent of strain *dna2-2*, strain 7585-2-2, (Formosa and Nitiss 1999), is not shown for simplicity but the average life span was determined and is 26.55± 13.91 (N=31).

Figure 1B. Dna2p overexpression strains have normal or slightly shortened life spans. Average life spans (number dissected in parentheses): UCC3515, *DNA2*$^+$, 22.9±5.9 generations (N=39); UCC3515 *DNA2*$^+$/p*Gal-CEN-DNA2*, 18.9±8.5 generations (N=37). Life span was determined in raffinose plus galactose medium.

Figure 1C. *DNA2* and *SGS1* are not epistatic. The strain background for this experiment was W303(*RAD5*) and all strains are isogenic (except for the *dna2* or *sgs1* alleles). Average life spans (number dissected in parenthesis): W303 *DNA2*$^+$ (wildtype), 24.3±10.2 generations (N=35); *dna2-2*, 7.3±3.8 generations (N=22); *sgs1Δ*, 14.7±10 generations (N=22); *dna2sgs1Δ*, 3.2±1 generations (N=22).

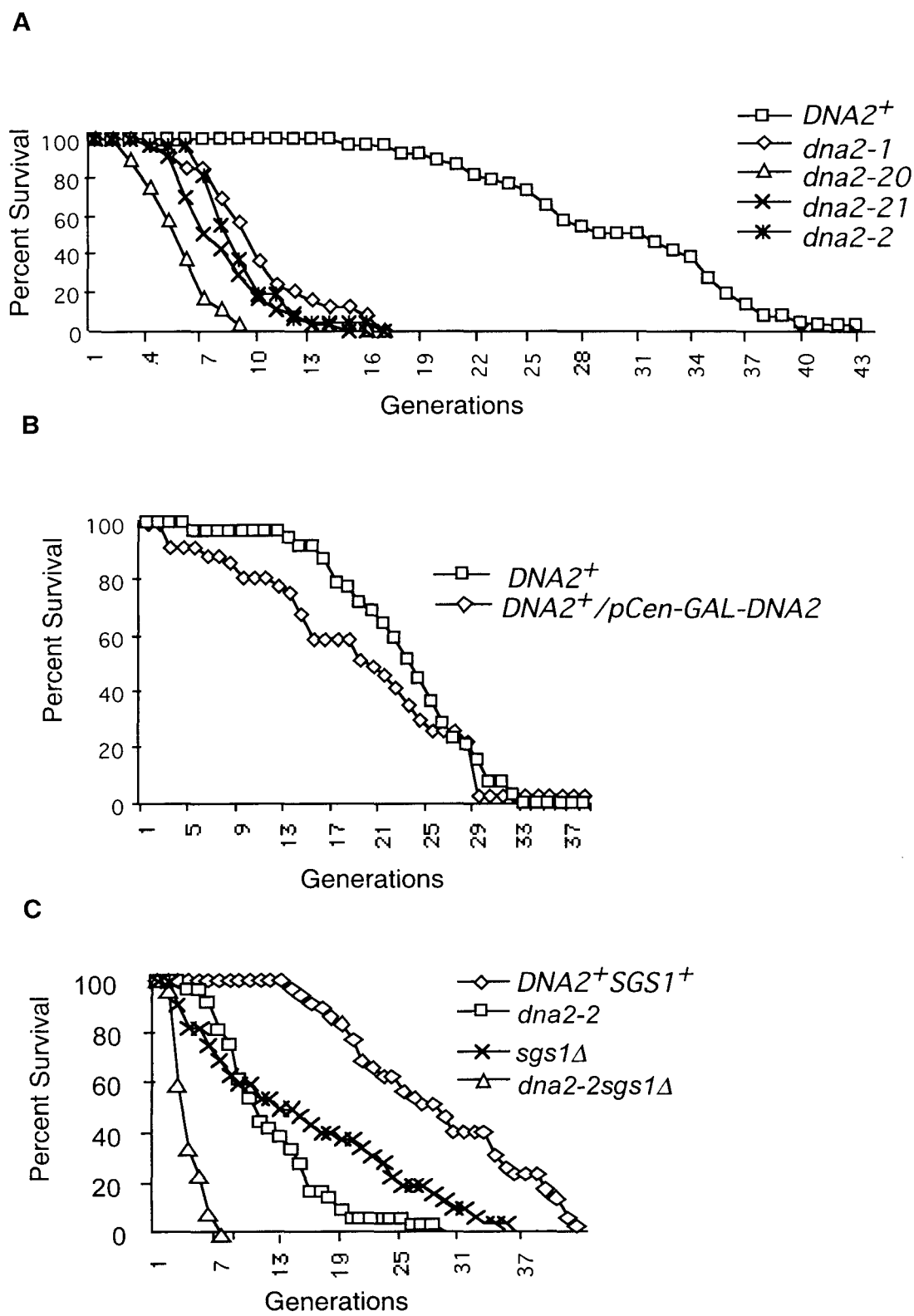

A
Percent Survival
100
80
60
40
20
0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
Generations
DNA2+
dna2-1
dna2-20
dna2-21
dna2-2
B
Percent Survival
100
80
60
40
20
0
1
5
9
13
17
21
25
29
33
37
Generations
DNA2+
DNA2+/pCen-GAL-DNA2
C
Percent Survival
100
80
60
40
20
0
1
7
13
19
25
31
37
Generations
DNA2+SGS1+
dna2-2
sgs1Δ
dna2-2sgs1Δ

Figure 1

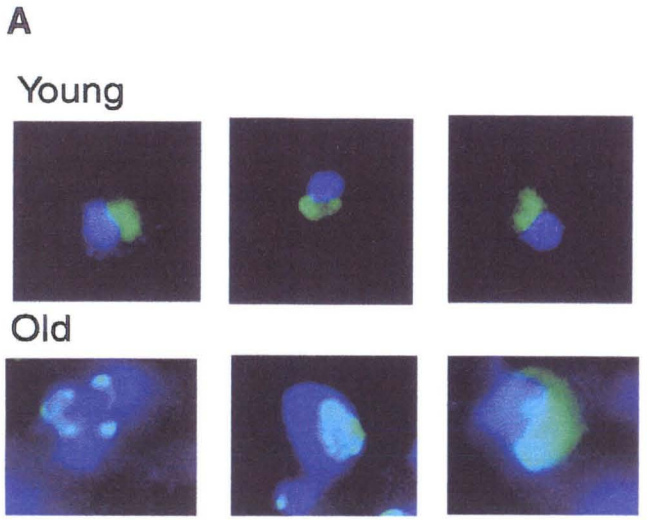
Figure 2A. Nucleolar enlargement and fragmentation accompanies aging in *dna2-2* strains. Both young cells (labeled YOUNG) and cells isolated eight generations after labeling for one generation with biotin (labeled OLD) were examined (see Materials and methods). Nuclei stained with DAPI (dark blue) and with anti-Nop1 antibody (green) were observed in young and in eight-generation *dna2-2* cells as indicated. The three cells shown are representative of >90-95% of the cells observed in each population, and as previously shown, do not represent simply dead cells (Sinclair et al. 1997).

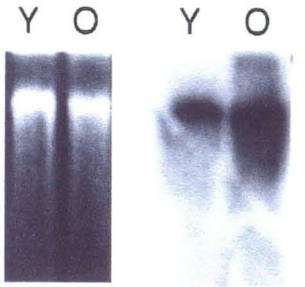
Figure 2B. rDNA is amplified in old cells derived from *dna2-2* mutants. *dna2-2* cells were prepared at zero and eight generations and DNA isolated as described (Sinclair and Guarente 1997). Total, undigested DNA was analyzed by conventional gel electrophoresis (left) and Southern blotting (right). The EtdBr stained gel is shown on the left, with DNA from equal numbers of zero- generation and eight-generation (O) cells (as determined by hemocytometer counting). As shown on the right, this gel was blotted to nitrocellulose and hybridized to an rDNA probe labeled by PCR of a plasmid. The upper band (greater than 15 kb) is composed of total genomic DNA. Circular rDNA (5kb) would migrate within or below the smear of degraded DNA. Wildtype (not shown) did not show amplification after eight generations.

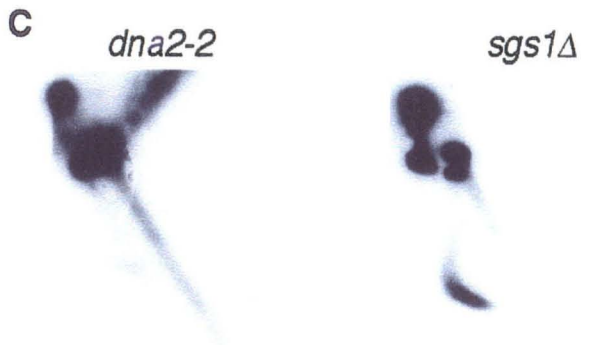
Figure 2C. rDNA isolated from *dna2-2* mutants (seven to eight-generations) contain extrachromosomal rDNA circles, but fewer than age-matched *sgs1Δ* cells.

Chromosomal DNA was prepared as described in Fig. 2B. Agarose gel electrophoresis was carried out in two dimensions, with each dimension containing chloroquine as described in Material and Methods. Southern blotting was carried out as in Fig. 2B. The DNA on the diagonal represents linear DNAs. DNA forming an arc to the left and below the diagonal is composed of closed circular topoisomers.

Figure 2D. ChIP assays in synchronized cells demonstrate that Dna2p associates with rDNA maximally during S phase. ChIP assays were performed using a strain in which the *DNA2* open reading frame was precisely replaced by *DNA2* fused to 9 myc tags in strain W303*pep4bar1*, such that the fusion protein is expressed under the control of its endogenous promoter (Choe and Campbell, unpublished). The strain shows the same growth rates as the parental strain and is designated DNA2TMTH. Crosslinking, cell lysis, shearing, immunoprecipitation, and PCR amplification were carried out as described in Materials and Methods. The DNAs amplified from the anti-myc 9E10 monoclonal antibody immunoprecipitates are labeled "IP" and correspond to DNAs associated with Dna2p. The PCR products 1 and 2 correspond to the rDNA regions labeled 1 and 2 in the diagram. Control PCR reactions using as template the total genomic DNA in the extracts showed that each fragment amplified equally at each time point (labeled INPUT). Single copy ARS1 probe showed no signal, and there is no internal control for rDNA, which is very high copy number. The data shown are from a 25 cycle amplification (see Materials and methods). The same procedure, carried out on a strain in which *DNA2* was not fused to myc, yielded no PCR product (not shown).


A
Young
Old


B
Y O Y O


C
dna2-2
sgs1Δ

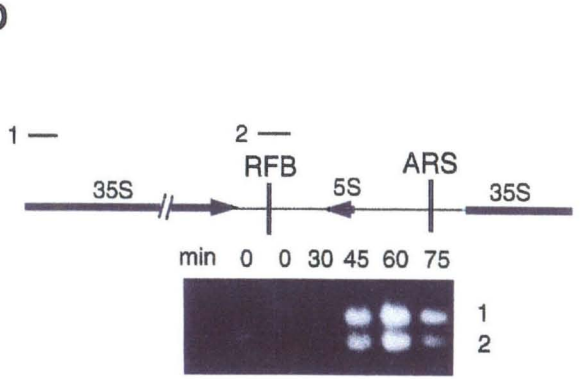

D
1
2
RFB
ARS
35S
5S
35S
min 0 0 30 45 60 75
1
2

Figure 3. Many, but not all, DNA replication mutants show reduced life span. Average life span (number dissected in parentheses): *pol1-14*, 11.5±8.6 generations (N=36); *pol1-17*, 21.2±7.6 generations (N=35); *rth1/rad27Δ*, 11.6±7.0 generations (N-32); *ctf4Δ*, 6.4±4.4 generations (N=31); *DNA2*$^{+}$ is SS111, 30.4±5.4 generations (N=37). The isogenic parental strain for the *pol1* and *rad27* mutants is SS111, see Fig. 1. *ctf4* is isogenic to the *dna2-2* strain shown in Fig. 1A that has an average life span of 8.2± 2.5 generations (N=33), and to the wild type strain 7585-2-2 (Tim Formosa), 26.55±13.91 generations, (N=31).

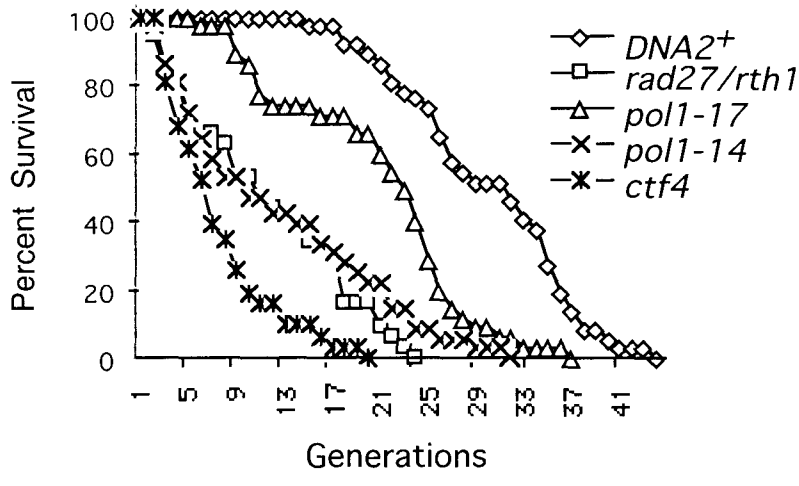

Percent Survival
100
80
60
40
20
0
1
5
9
13
17
21
25
29
33
37
41
Generations
DNA2+
rad27/rth1
pol1-17
pol1-14
ctf4

Figure 3