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

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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 dna2Δ−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 XDna2p 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 (ScDNA2)² is essential for viability. ScDNA2 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 another 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 Scdna2−1 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 semi-conservatively 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 G₁ 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 ScDNA2 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 dna2Δ−1 mutant, and similarly overproduction of ScDna2p suppresses the temperature-sensitive growth of a rad27Δ−1 mutant. Second, dna2α−1 and rad27Δ−1 alleles are synthetically lethal. Third, certain rad27 mutants require overproduction of the G₁ 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-
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 supported 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 Cts4p.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 mutant fails 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 ScDNA2, whose characterization we report here. The XDna2 gene is probably a functional homolog of ScDNA2, since XDna2 efficiently complements an ScDNA2 mutant for growth at the permissive 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, Caeorhabditis elegans, and S. cerevisiae Dna2 proteins (motifs 2 and 3; Fig. 1): primer A, ATCGAATTCACGATCCCCGGCGCTTGGGA/TCAATA(T/C)ATI(T/C)/T; primer B, AGGAAGCTTGCCAC/AGTTAACA/GCCGG-C/G(T/C)ATI(G/C). The extra nucleotides, underlined in each primer, provide restriction sites for EcoRI or HindIII. 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 EcoRI/HindIII, and cloned into plasmid LITMUS39 (New England Biolabs) to give plasmid pEHPROBE. The EcoRI/HindIII fragment of pEHPROBE was radio-labeled 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 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 XDna2p gene. The XDna2 gene was cloned into pFASTBacHTa in the following procedure. The small Ehe/EcoRI fragment in the polynucleotide site of pFASTBacHTa was replaced by a linker containing a NdeI site to give plasmid pFASTNdeI; the XDna2 C-terminal fragment from the EcoRI to the Ma1 site was inserted into the EcoRI site of plasmid pETXDna2, to give pETXDna2; the XDna2 gene; the NdeI/XhoI fragment containing the XDna2 gene from pETXDna2 was inserted into NdeI/XhoI sites of pPASTNde1 to give plasmid pPASTXDna2. pPASTXDna2 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$_x$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 mM 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 mM NaCl, 5 mM imidazole) for 1 h at 4 °C. The resin was washed with 4 ml of binding buffer and subsequently 4 ml of washing buffer (20 mM Tris-HCl pH 7.9, 0.5 mM NaCl, 60 mM imidazole). DNA2p was eluted with 150 μl of buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 300 mM imidazole, and 10% glycerol). All of the buffers except the elution buffer contain protease inhibitors: phenylmethylsulfonl 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 BamHI/SalI fragment containing the C-terminal 2.8 kilobase pairs of the XDna2 gene from pETXDna2 was cloned into BamHI/SalI sites of pSEY18-GAL (20) to give plasmid pSEYBS. To clone the remaining 1.0 kilobase pairs into BamHI site in pSEYBS equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 60 mM imidazole) for 1 h at 4 °C. The resin was washed with 4 ml of binding buffer and subsequently 4 ml of washing buffer (20 mM Tris-HCl pH 7.9, 0.5 mM NaCl, 60 mM imidazole). DNA2p was eluted with 150 μl of buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 300 mM imidazole, and 10% glycerol). All of the buffers except the elution buffer contain protease inhibitors: phenylmethylsulfonl fluoride (1 mM), pepstatin (2 μg/ml), leupeptin (1 μg/ml), and benzamidine (2 mM).

Preparation of Radiolabeled Substrates for DNA Helicase and Nuclease Assays—A 44-mer oligodeoxynucleotide (AGCTTTGATCCTAGGTTCAGGACGCTTGGAGAGTTGGTGA/GTGGAACTATGAAACCCGACA/GCCCGTGAAGTCCGCGGCCAGCAC) was labeled at the 3' end using polyuridylic 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 unincorporated oligonucleotide were removed by gel filtration using Cibacron Blue 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 [γ-$^32$P]ATP. Annealing buffer was added to the 3'-labeled substrate to 100 μl. Gel filtration removed unincorporated [γ-$^32$P]ATP. The filtrate (3 μl) was used directly for the nuclease assay.
A Sequence of XDna2

- Nterminus: 1-30
- Cterminus: 1023-1053

B Homology Among XDna2, HDna2, CDna2, ScDNA2 and A DNA2:

| Species       | Length (aa) | Identity |
|---------------|-------------|----------|
| Xenopus laevis| 1053        | 100%     |
| Human         | 1053 (partial) | 63%     |
| Canmorhildia elegans | 1106 | 33% |
| Staphylococcus aureus | 1521 | 33% |
| Arabidopsis thaliana | 1286 | 37% |

C Conserved motifs among DNA2 homologs

| Part       | Motifs | Conserved |
|------------|--------|-----------|
| Part 1     | 105    | C, F      |
| Part 2     | 120    | C, F      |
| Part 3     | 151    | C, F      |
| Part 4     | 119    | C, F      |
| Part 5     | 127    | C, F      |

D Nucleotide Sequence—Purified XDna2p was incubated with substrate (0.075 pmol of oligonucleotide) in 20 μl of reaction buffer (40 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 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 15% sequencing gel. The gel was run at 200 V for 150 min. Gels were then dried and analyzed using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

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

F RESULTS

Cloning of the XDna2 Gene—A data base search for protein sequences similar to ScDNA2 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 ScDNA2. 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 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. The sequence is similar to ScDNA2 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. The sequence is similar to ScDNA2 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. The sequence is similar to ScDNA2 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.

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 2–8 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, A DNA2p, 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.

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 the DNA2 gene. C. elegans is conserved from yeast to human. Motif 1 contains the proline residue that corresponds to the mutated proline in the DNA2 gene.
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. 1B). A recent data base search revealed another Dna2 homolog (GenBank™ AC0003981 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 CDna2p revealed many conserved residues over the entire length of the protein. In Fig. 1C, 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. 1A) within domains III–V. The proline residue in motif 1 of domain 1 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. 1A 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. 2A, lanes 2 and 4). We conclude that this is the XDna2 gene product, because it migrates 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.

**FIG. 2. Detection of endogenous XDna2p and purification of recombinant His<sub>6</sub>-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 N-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<sub>6</sub>-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<sub>6</sub>-XDna2p.

Lane 1, crude extract; lanes 2–4, 10, 25, and 50 ng of purified protein, respectively.

**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 2 and 1, respectively, but streaked after growth on 5-fluoroorotic acid plates, which select for isolates that have lost the Ura<sup>+</sup> plasmids. pGALScDna2 was a construct in which the ScDNA2 gene was cloned into pSEY18-GAL.

**CDna2p revealed many conserved residues over the entire length of the protein. In Fig. 1C, 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. 1A) within domains III–V. The proline residue in motif 1 of domain 1 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. 1A are the two conserved motifs used to design the two degenerate primers for PCR.)**

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**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, GALL,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
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 ScDNA2 (Fig. 3, compare sectors 1 and 5). Overproduction of ScDNA2 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 ScDNA2.

**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. His6-XDna2p was purified by Ni²⁺/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. 2B.

Purified His6-XDna2p had ATPase activity (Fig. 4A, lanes 5–7, and Fig. 4C) that was greatly stimulated by poly(dA) (Fig. 4A, lanes 5–7, and Fig. 4C).
The optimum concentration of poly(dA) was 1.5 mM (Fig. 4B, 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, His6-XDna2K655E, 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 His6-XDna2K655E mutant preparation had a barely detectable background of ATPase activity (Fig. 4A, lanes 2–4, and Fig. 4C), and this activity was not stimulated by DNA (Fig. 4B, lanes 1–5, and Fig. 4D). Thus, we conclude that the ATPase observed in the His6-XDna2p preparation is due to XDna2p. The ATPase of XDna2p 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

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4B, lanes 6–10, and Fig. 4D). The optimum concentration of poly(dA) was 1.5 mM (Fig. 4B, 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, His6-XDna2K655E, 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 His6-XDna2K655E mutant preparation had a barely detectable background of ATPase activity (Fig. 4A, lanes 2–4, and Fig. 4C), and this activity was not stimulated by DNA (Fig. 4B, lanes 1–5, and Fig. 4D). Thus, we conclude that the ATPase observed in the His6-XDna2p preparation is due to XDna2p. The ATPase of XDna2p 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

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5 W. Choe and J. L. Campbell, unpublished results.
oligonucleotide with a 14-nucleotide noncomplementary 5' tail (Fig. 5A, 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. 5A, 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. 5A, 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 His6-XDna2p was also expressed and purified. However, no nuclease activity was detected (Fig. 5A, lanes 1–3), and therefore the location of the nuclease catalytic site remains unknown. From Fig. 5A, 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, a 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. 5B). The major products were oligomers of 5, 6, and 7 nucleotides (Fig. 5B, 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. 5B, 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-32P]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. 5C). The products from XDna2p digestion appeared as one band (the bands of >32 nt in lanes 7–9, Fig. 5C), 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. 5D, digestion of circular, single-stranded M13 DNA by His6-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. 5C) was incubated with purified XDna2p in the presence of different concentrations of XDna2p antiserum or preantiserum (Fig. 5E). 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. 5B 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.

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' nucleases was detected (see Fig. 5C). 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 XDna2p, 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 XDna2p present in the extracts. From Western blot analysis of the immunoprecipitate and the supernatant after depletion, we conclude that XDna2 antibody effectively immunoprecipitates XDna2p but that mock depletion does not (Fig. 6).

**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 His6-XDna2p 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.
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. 7A, 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. 7C, 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 antisera 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. 7D). 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 Sdna2-1 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...
sequences from human, *C. elegans*, and *Arabidopsis* in the database 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/μg, 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 XDna2p 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 RecBC,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. Mutation of *mcm10* suppresses mutations in *mcm2–7* genes and is thought to be involved in initiation (18). 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. 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.

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

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**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. 7B. 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.
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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