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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 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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[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 pETXDna2 was inserted into *Nde*I/*Xho*I sites of pFASTNdeI to give pFASTXDna2. 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.

A Sequence of XDna2

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   1  MEPVSAECHL PPEDDLLEMM MEQSFTEPEE KSQDKPTRKI IPKTKLCKGV
  51  NNRYCVLHIK EVYAQREEKH LTITASQEGD DLELCILKDD WVALQIKPGD
 101  IIHLEGNCSV DNTWTISRDT GYLILYPDLL ISGTSIANGI RCLRRSVLSE
 151  KFKVCDKGSR QMLNGTMLHD IFQRATTCGF TDSVLQELAH HTVHGPKYLK
 201  EMYQLKLNQA DVMGEIQEYL PSLSKWATDF MTHPLNQQQI NRTKSTAGDP
 251  TETTKVSEFL DIEENIWSPR FGLKGKIDVT ARVKIHQKSK AHLKIMPLEL
 301  KTGKESNSIE HRSQVVLYTL LSQERREDPE AGLLLYLKTG NMYTVPGNRL
 351  DRRELLKIRN ELSYYLTNVL HKSDNGSKET TLASLPAMIA DRQACKYCSQ
 401  MRNCALYNRS VEQQTENCYI PPEMIPVVQK ETEHLTEDHL QYFRLWYLMC
 451  TLEANSKDSK MGRKNIWMMS SSEREEDGQC IGNLIRTGHV QTISDVQYLH
 501  SFQRRSGSVP ATNLASGDRV VVSGEERFLA LSTGYIKEVK DENITCILDR
 551  SLVKLPEDLL FRLDHEEGGG GLEFHLGNLS RLMENSSVSE KLRKLIIDFS
 601  KPNFVQHLSS ILPPDAKDIV ASILRGLNKP QKQAMKRVLL SKDYTLIVGM
 651  PGTGKTTTIC TLVRILYACG FSVLLTSYTH SAVDNILLKL KKFQVGFLRL
 701  GRTQKLHPDV QEFSEEEICK AKSIKSLSAL EELYNSQPVV ATTCMGVNHP
 751  IFTRRRFDFC IVDEASQISQ PICLGPLFFA DRFVLVGDHQ QLPPLVKSAE
 801  ARELGMSESL FKRLERNQEA VVQLTVQYRM NSKIMALSNK LVYEGRLECA
 851  SDRVSNAVVQ LPHIKTLLLE LEFRESQESM WIKDVLEPSN PVCFLNTEKI
 901  PALETEEKGG ISNWIEAKLV FHLTKLYLKA GCRPSDIGII APYRQQLKMI
 951  SNYFNSLSAS AVEVNTVDKY QGRDKSVIIV SFVRSNIDGK LGDLLKDWRR
1001  LNVALTRAKH KLIMLGCVPT LNRFDCLEQL ICNLKTENQI YDLPEGAHEH
1051  FPV*
```

B Homology Among XDna2, HDna2, CDna2, ScDna2 and ADna2:

Species	Length (aa)	Identity
Xenopus laevis	1053	100%
Human	1076 (partial)	63%
Caenorhabditis elegans	1106	33%
Saccharomyces cerevisiae	1521	34%
Arabidopsis thaliana	1286	37%

C Conserved motifs among Dna2 homologs

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Part 1:
XDna2   120 .GYLILYPDL LISGTSIANG IRCLRRSVLS EKFKVC.DKG SRQMLNGTML HDIFQRATTC
HDna2       .GYLILYPDM LISGTSIASS IRCMRRAVLS ETFRSS.DPA TRQMLIGIVL HEVFQKAINN
ScDna2  496 DMLLVLNPDV LFSATSVGSS VGCLRRSILQ MQFQDPRGEP SLVMTLGNIV HELLQDSIKY
ADna2   391 NNFLIVHPDT LVAGTRVAAS FGCPRRTVLD ERLRS..NEH ATVALLGYLQ HQWFQAGLSQ
CDna2   203 .GIVILNPDA LVPCTAVASA TFCARKTVLN DKFKFG.NAS NKAMLLGTIM HEIFQTAITS
Consensus   ---LIL-PD- L---T--A-- --C-RR-VL- E-F------- ---ML-GT-- HE-FQ
                      1

Part 2:
XDna2   260 DIEENIWSPR FGLKGKIDVT ARVKIHQKSK A.HLKIMPLE LKTGKESN.. .SIEHRSQVV LYTLLSQERR
HDna2       DIEESIWSPR FGLKGKIDVT VGVKIHRGYK T.KYKIMPLE LKTGKESN.. .SIEHRSQVV LYTLLSQERR
ScDna2  639 DIEENIWSPI YGLKGFLDAT VEANVENNKK ....HIVPLE VKTGK.SR.. .SVSYEVQGL IYTLLLNDRY
ADna2   527 DIEEMSWAPK YGLKGMIDAS VRVIVESDMN TVNEKIMPLE FKSGKAPSGQ SSIEHSAQVI LYTLLMSERY
CDna2   338 DIEENIWDSK LGLKGKIDVT MRTKSRGGME .......SLE LKTGKSS... CSSEHTCQVL LYCMMQSSRY
Consensus   DIEE-IW-P- -GLKG-ID-T ---------- -----I-PLE -KTGK-S--- -S-EH--QV- LYTLL--ER
                    2                                        3

Part 3:
XDna2   626 LNKPQKQAMK RVLLSKDYTL IVGMPGTGKT TTICTLVRIL YACGFSVLLT SYTHSAVDNI LIKLKKFQVG ..FLRLGRI
HDna2       LNKPQRQAMK KVLLSKDYTL IVGMPGTGKT TTICTLVRIL YACGFSVLLT SYTHSAVDNI LIKLAKFKIG ..FLRLGQI
ScDna2  1051 LNLNQKEAID KVMRAEDYAL ILGMPGTGKT TVIAEIIKIL VSEGKRVLLT SYTHSAVDNI LIKLRNTNIS ..IMRLGMP
ADna2   895 LNNDQRQAIL KILTAKDYAL ILGMPGTGKT STMVHAVKAL LIRGSSILLA SYTNSAVDNL LIKLKAQGIE ..FLRIGRI
CDna2   685 LNNEQRKAVV HALATEDFMM VEGLPGSGKT TLISVLIQCL VATNKKVLLA AFTHSAVDNI LTKLTKEVAA EKILRLGSS
Consensus   LN--Q--A-- --L---DY-L I-GMPGTGKT T-I------L ---G--VLL- SYTHSAVDNI L-KL------ ---LRLG--
                                                4

Part 4:
XDna2   733  YNSQPVVATT CMGVNHPIFT ..RRRFDFCI VDEASQISQP ICLGPLFFAD RFVLVGDHQQ LPPLVKSAEA
HDna2        YNSQLIVATT CMGINHPIFS ..RKIFDFCI VDEASQISQP ICLGPLFFSR RFVLVGDHQQ LPPLVLNREA
ScDna2  1154 INSTSVVATT CLGINDILST LNEKDFDYVI LDEASQISMP VALGPLRYGN RFIMVGDHYQ LPPLVKNDAA
ADna2   997  LDQVKVVAST CLGINSPLLV ..NRRFDVCI IDEAGQIALP VSIGPLLFAS TFVLVGDHYQ LPPLVQSTEA
CDna2   795  MKTTPIVACT CHHVPRELL. FSYRMFDVVI VDEASHVLEP LLLPVIATSN KFVLVGDCKQ LTPLVVSRKA
Consensus    -------VA-T C-G-N----- -----FD--I -DEASQI--P --LGPL---- -FVLVGDH-Q LPPLV----A
                                                5                   6

             RELGMSESLF KRLERNQEA VVQLTVQYRM NSKIMALSNK LVYEGRLECA
             RALGMSESLF KRLEQNKSA VVQLTVQYRM NSKIMSLSNK LTYEGKLECG
             RLGGLEESLF KTFCEKHPES VAELTLQYRM CGDIVTLSNF LIYDNKLKCG
             RENGMGISLF RRLSEAHPQA ISVLQNQYRM CRGIMELSNA LIYGDRLCCG
             KQEGAGISTM EKLQQSHPGV VVSLISQYRM NRETSVLSSK LFYENRLICG
             R--G---SLF --L-E----- V--LT-QYRM ---I--LSN- L-YE--L-CG

Part 5:
XDna2   932  RPSDIGIIAP YRQQLKMISN YFNSLSASAV EVNTVDKYQG RDKSVIIVSF VRS..NIDGK
HDna2        SPSDIGIIAP YRQQLKTIND LLAR.SIGMV EVNTVDKYQG RDKSIVLVSF VRS..NKDGT
ScDna2  1356 PCEDIGVMTL YRAQLRLLKK IFNKNVYDGL EILTADQFQG RDKKCIIISM VRR..NSQLN
ADna2   1148 DSKDIGIITP YNSQASLIQH AI...PTTPV EIHTIDKYQG RDKDCILVSF VRSREKPRSS
CDna2   996  KPHEIGVMSA YRRQVDHIRG ILNS...DEL EVNTIDSYQG REKRVIIWSL TWT..NNSTK
Consensus    ---DIG---- YR-Q---I-- ---------- EV-T-D-YQG RDK---I--S- VR---N----
                                                7

             LGDLLKDWRR LNVALTRAKH KLIMLGCVPT LNRFDCLEQL
             VGELLKDWRR LNVAITRAKH KLILLGCVPS LNCYPPLEKL
             GGALLKELRR VNVAMTRAKS KLIIIGSKST IGSVPEIKSF
             ASSLLGDWHR INVALTRA.. KLIMVGSQRT LSRVPLLMLL
             KSELLKDERR VNVALTRARQ KLVVVGCKNS AETIPVLFRF
             ---LLKD-RR -NVALTRA-- KLI--G---- -----P-L---
                       8
```

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

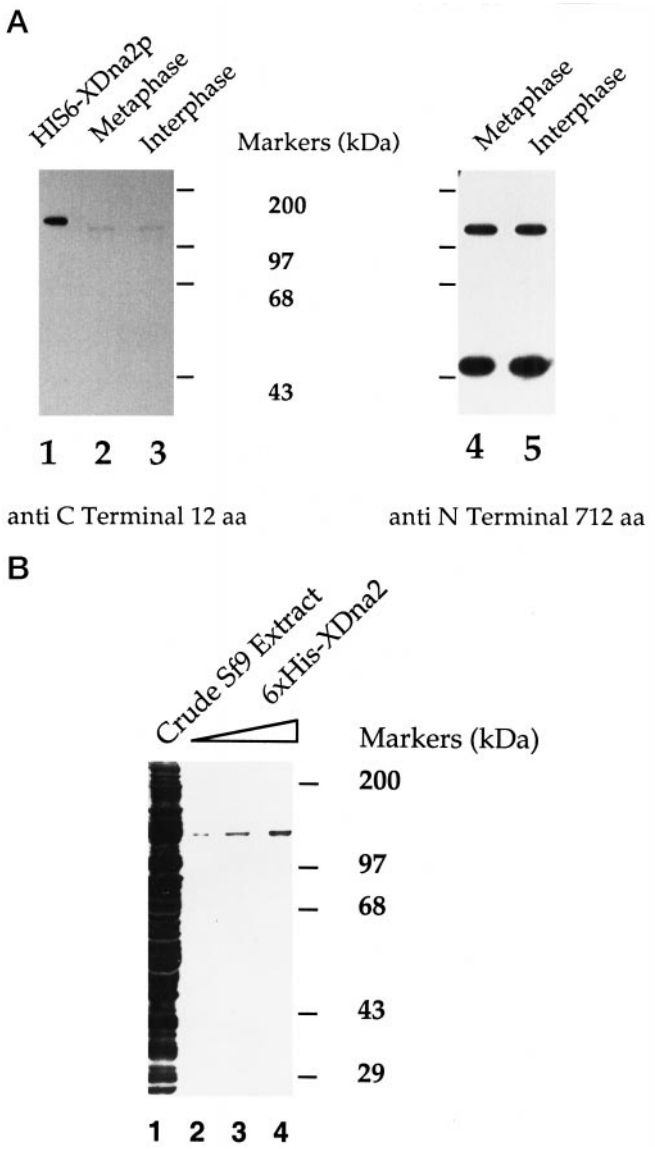

FIG. 2. **Detection of endogenous XDna2p and purification of recombinant His_6-XDna2p from insect cells.** *A*, detection of endogenous XDna2p from egg extracts by Western blotting. Proteins in metaphase and interphase egg extracts were separated by SDS-polyacrylamide gel electrophoresis. Gels were blotted 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

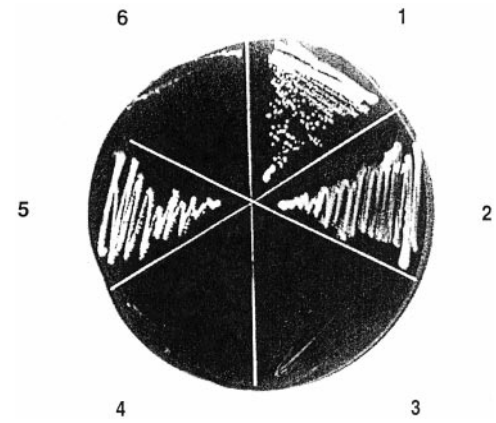

FIG. 3. **The XDna2 gene can complement *S. cerevisiae dna2-1*.** Plates carrying either wild type or *dna2-1* mutant yeast transformed with various plasmids were incubated at 37 °C. *Sectors 1*, *5*, and *6*, *dna2-1* transformed with pGALXDna2, pGALScDna2, or pSEY18-GAL, respectively; *sector 2*, wild 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 *S*cDna2p (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

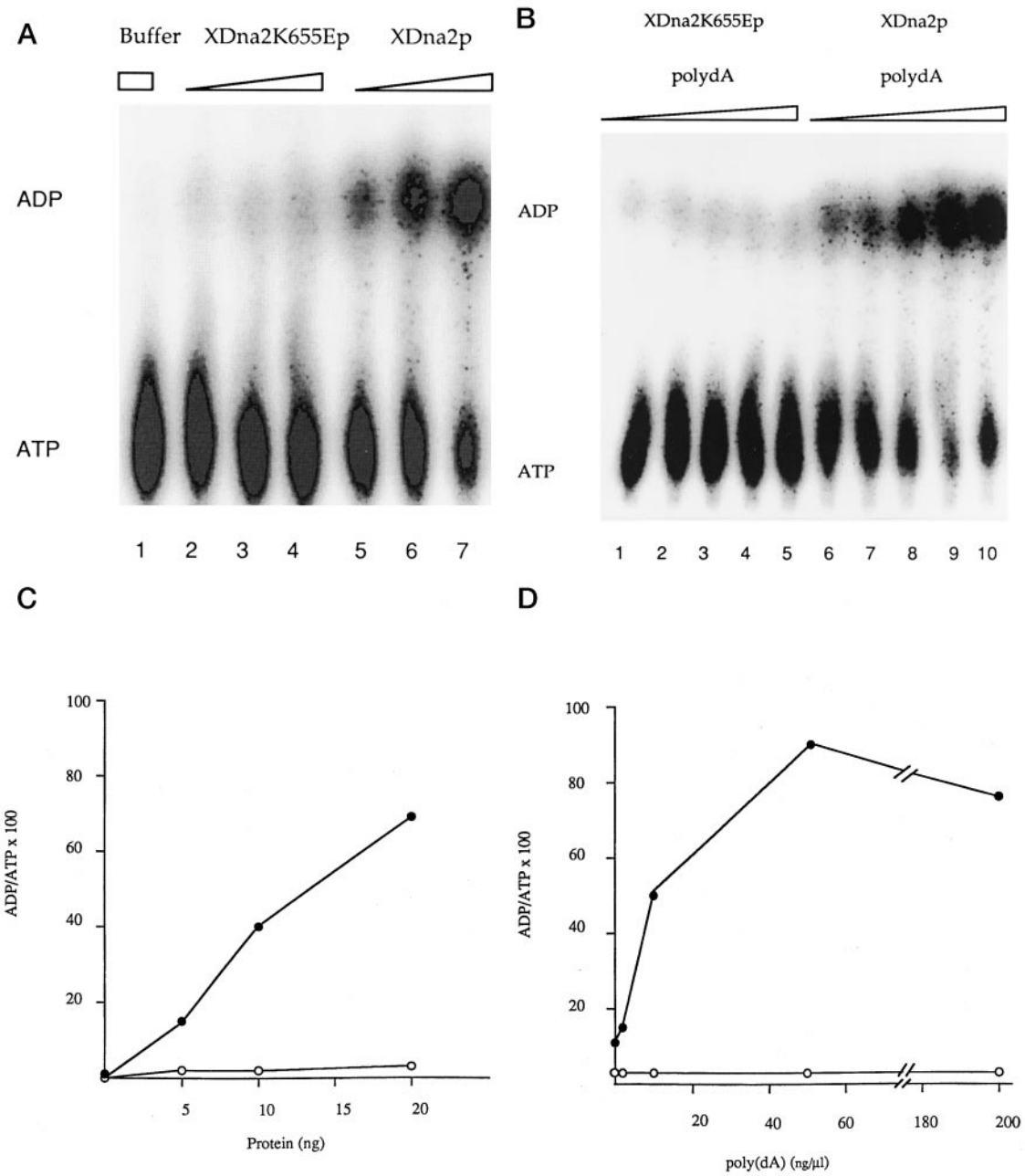

FIG. 4. **XDna2p is a DNA-dependent ATPase.** Purified His_6-XDna2p or His_6-K655Ep was incubated with [α-^{32}P]ATP for 1 h at 37 °C in a buffer described under "Experimental Procedures." The products were spotted onto a polyethyleneimine-cellulose plate that was then developed in 0.5 M LiCl, 1.0 M 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.

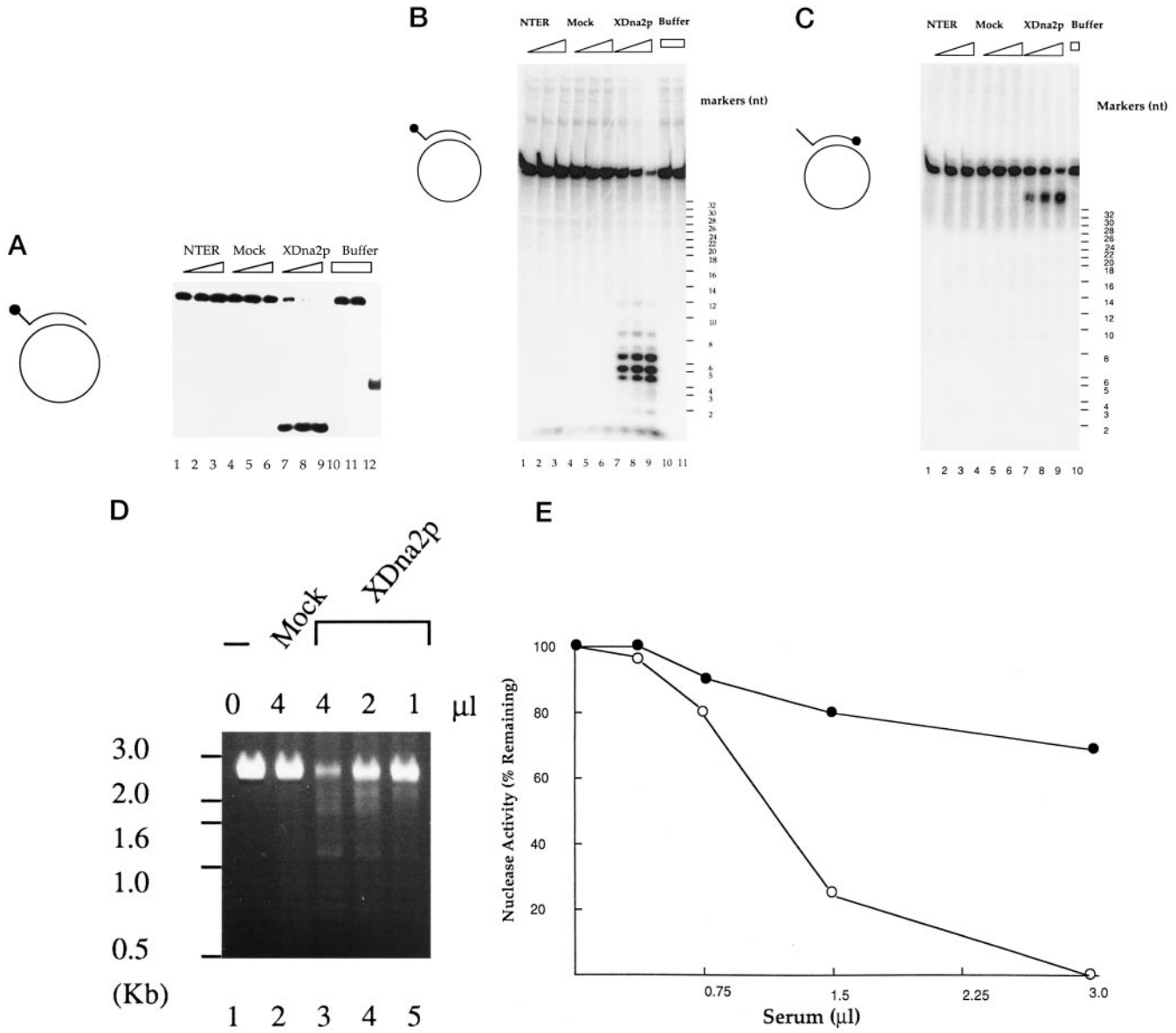

FIG. 5. **XDna2p is a single-stranded DNA endonuclease.** *A*, helicase assay reveals nuclease activity only. A 5′-end-labeled oligonucleotide annealed with M13 ssDNA (shown on the *left*) was incubated with increasing concentrations of His_6-NTERp (N-terminal 556-amino acid fragment of XDna2p) (*lanes 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.

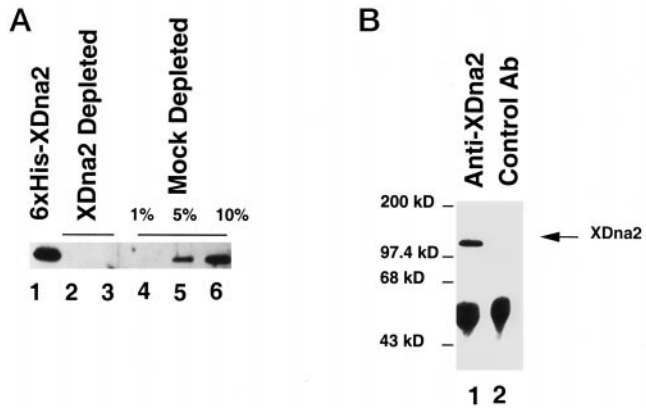

FIG. 6. **Immunodepletion of XDna2 from interphase extracts.** 20 μl of protein A beads were incubated with 20 μg of either affinity-purified anti-XDna2 antibody or a control antibody for 1 h at room temperature and then were washed with 1× HBS buffer. 20 μl of interphase extract was incubated with 10 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

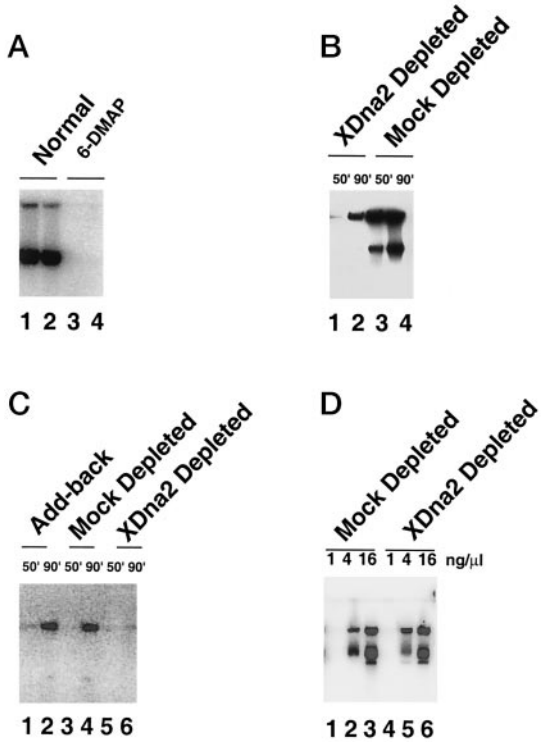

FIG. 7. **Chromosomal DNA replication is inhibited in XDna2-depleted extracts.** Extracts were prepared as described under "Experimental Procedures." *A*, 6-DMAP can inhibit DNA replication in extracts. 10 μl of normal interphase extract (*lanes 1* and *2*) or 6-DMAP-treated extract (*lanes 3* and *4*) 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 Sc*dna2-1* 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

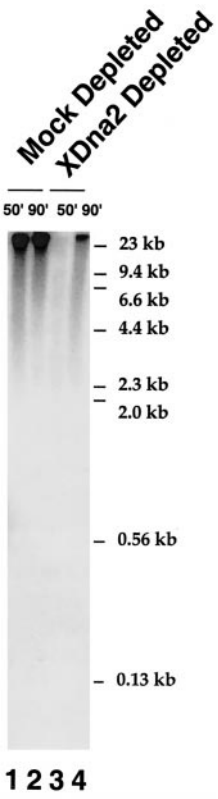

FIG. 8. **Analysis of the DNA replication products from the XDna2-depleted or mock-depleted extracts after denaturation on an alkaline agarose gel (1%).** The preparation of DNA replication products is the same as described in the legend to Fig. 7*B*. *Lanes 1* and *2*, DNA products synthesized in mock-depleted 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 *rad27*Δ, 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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