

Biochemical analysis of human Dna2

Taro Masuda-Sasa, Osamu Imamura and Judith L. Campbell*

Braun Laboratories, 147-75, California Institute of Technology, Pasadena, CA 91125, USA

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ABSTRACT

Yeast Dna2 helicase/nuclease is essential for DNA replication and assists FEN1 nuclease in processing a subset of Okazaki fragments that have long single-stranded 5' flaps. It is also involved in the maintenance of telomeres. *DNA2* is a gene conserved in eukaryotes, and a putative human ortholog of yeast *DNA2* (ScDNA2) has been identified. Little is known about the role of human *DNA2* (*hDNA2*), although complementation experiments have shown that it can function in yeast to replace ScDNA2. We have now characterized the biochemical properties of hDna2. Recombinant hDna2 has single-stranded DNA-dependent ATPase and DNA helicase activity. It also has 5'–3' nuclease activity with preference for single-stranded 5' flaps adjacent to a duplex DNA region. The nuclease activity is stimulated by RPA and suppressed by steric hindrance at the 5' end. Moreover, hDna2 shows strong 3'–5' nuclease activity. This activity cleaves single-stranded DNA in a fork structure and, like the 5'–3' activity, is suppressed by steric hindrance at the 3'-end, suggesting that the 3'–5' nuclease requires a 3' single-stranded end for activation. These biochemical specificities are very similar to those of the ScDna2 protein, but suggest that the 3'–5' nuclease activity may be more important than previously thought.

INTRODUCTION

Dna2 is a multifunctional enzyme, conserved in eukaryotes, and involved in various aspects of DNA metabolism. *Saccharomyces cerevisiae DNA2* (ScDNA2) is essential, and involved in DNA replication, DNA repair, telomere biogenesis and stable maintenance of ribosomal DNA (1–7). The function of ScDna2 in DNA replication has been especially well studied. ScDNA2 interacts genetically and physically with proteins involved in lagging-strand DNA synthesis, such as flap endonuclease (FEN1) and Exo1, and RNaseH2 (1,8–11). The *dna2-1* mutant is temperature sensitive and cannot

synthesize high molecular weight DNA at restrictive temperatures (5). Several lines of biochemical evidence indicate that ScDna2 is involved in primer removal during Okazaki fragment maturation in cooperation with FEN1 and RPA. ScDna2 possesses 5'–3' helicase activity specific for a forked substrate, single-stranded DNA-dependent ATPase, and 5'–3' exo-endonuclease activity specific to single-stranded DNA (5,6,12). The nuclease activity of ScDna2 prefers the 5' tail of flap structure such as might be produced as a result of primer displacement during Okazaki fragment maturation. Binding of RPA to flap substrates with a long tail inhibits the nuclease activity of FEN1, but stimulates the helicase/nuclease activity of ScDna2, and maturation of the lagging-strand needs both FEN1 and Dna2 (13–17). It has been proposed that ScDna2 processes an RPA-coated, long flap structure that is a poor substrate for cleavage by FEN1, resulting in short flaps that are optimum substrates for FEN1. In the process of flap removal, Dna2 employs a tracking mechanism that requires the recognition of the free 5'-terminus and then movement to the base of the flap for cleavage (18). Moreover, ScDna2 has a role in the pathway for the processing of structured flaps, in which it aids FEN1 using both its nuclease and helicase activities (13,16). The nuclease activity of ScDna2 is suppressed in the presence of ATP, allowing the helicase to unwind double-stranded DNA before the action of nuclease (5–7,12,13). This coupling of the 5'–3' endonuclease activity and DNA helicase activity is thought to contribute to processing of structured flaps. Consistent with the role of Dna2 in flap processing, reduced strand displacement reduces the need for DNA2, while increased strand displacement and decreased ability to idle at a nick, increases the need for DNA2 *in vivo* (8).

In addition to its well-studied role in Okazaki fragment processing, ScDna2 is involved in both telomerase-dependent and telomerase-independent telomere elongation pathways (3). The lethality of deleting the essential Dna2 helicase/nuclease from budding yeast is suppressed by deletion of *PIF1*, which encodes a DNA helicase involved in the suppression of telomere elongation (19–22). In accordance with its function in telomere maintenance and DNA replication, ScDna2 is localized to telomeres in G1 and G2 phase of the cell cycle, but relocalizes during S phase to internal sites in the chromosomes (3). With respect to ribosomal DNA, a mutant of ScDNA2 shows increased pausing at the rDNA replication fork barrier (RFB) and accumulates DSBs at the RFB in a

*To whom correspondence should be addressed. Tel: +1 626 395 6053; Fax: +1 626 449 0756; Email: jcampbel@caltech.edu

FOB1-dependent manner. Thus, ScDNA2 is involved in the maintenance of rDNA (23,24).

DNA2 orthologs are found in all other eukaryotes examined to date. Consistent with its role in *S.cerevisiae* DNA replication, in *Schizosaccharomyces pombe*, *dna2*⁺ is essential, genetically interacts with lagging-strand processing enzymes including FEN1 and DNA polymerase δ , and is involved in Okazaki fragment elongation/maturation (25). It also localizes to telomeres and is involved in telomere metabolism (26). In metazoans, *Caenorhabditis elegans* Dna2 protein (CeDna2) is a helicase/nuclease that can be stimulated by *C.elegans* RPA, and homologous deletion of CeDNA2 shows growth deficiency in a temperature-sensitive manner (27,28). CeDNA2 mutants show 90% embryonic viability in F1 but are embryonic lethal in F2, a phenotype of telomere deficiency in other organisms. *Xenopus* Dna2 is a nuclease/ATPase, and important for DNA replication in the cell-free DNA replication system of *Xenopus* egg extracts, and the XDNA2 gene complements yeast *dna2-1* mutants (29).

These reports suggest that functions of ScDNA2 are conserved in eukaryotes. However, little is known about the function of human DNA2 (hDNA2). We have shown that a putative human DNA2 ORF, DNAL, can complement the *dna2-1* temperature-sensitive mutant of ScDNA2, suggesting that hDNA2 is a functional ortholog of ScDNA2 (30). Although mutations in hDNA2 have not yet been directly associated with human disease, it is notable that the human WRN and BLM genes, which encode RecQ helicases, can suppress the lethality of yeast *dna2-1* mutations (30,31). This suggests that Dna2 may functionally interact with or play redundant roles with these helicases in maintaining telomeres and/or in suppressing excessive sister chromatid exchange, and that it is therefore important to investigate the human Dna2 protein and gene (32).

In this paper, we purified recombinant hDna2 protein (hDna2) from insect cells, and investigated its biochemical activity to learn the function of hDna2. hDna2 showed ATPase/helicase activity and 5'-3' exo-endonuclease as well as 3'-5' exo-endonuclease activity, indicating that its biochemical properties are very similar to those of other organisms.

MATERIALS AND METHODS

Proteins

hRPA. hRPA and details for its use were from Marc Wold (University of Iowa, Iowa City, Iowa).

hDna2. To produce recombinant hDna2, hDna2-Flag was excised from pRS316/GAL-hDNA2-Flag (30) with BamHI/XhoI and inserted into the corresponding sites of pFastBac HTc vector (Invitrogen). The nuclease-defective hDna2D294A point mutant was created using the QuikChange site-directed mutagenesis kit (STRATAGENE) using the primers 5'-GGATTGAAAGGCAAATAGCTGTTACAGTTGGTGTGAAATAC-3' and 5'-GTATTTTCACCAACTGTAAACAGCTATTTTGCCTTCAATCC-3' and confirmed by DNA sequencing. The helicase-defective hDna2K671E point mutation was created using the primers

5'-GGTATGCCTGGGACAGGAGAAACAACACTACGATATGTACTCTC-3' and 5'-GAGAGTACATATCGTAGTTGT-TTCTCCTGTCCCAGGCATACC-3' and was confirmed by DNA sequencing.

Baculovirus expressing hDna2 (wild type, D294A or K671E) was infected into High5 cells, and incubated using shaker flasks for 60 h at 27°C (MOI: 5, one liter culture). Infected cells were then harvested, and resuspended in 100–250 ml lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 20% glycerol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and COMPLETE protease inhibitor cocktail (Roche)]. Cells (~13 g) were lysed by sonication using BRANSON SONIFIER S-450A with microtip (Duty Cycle 35, Output 6, 10 s, three times), and cell lysates were clarified by centrifugation for 20 min at 10 000 g. The supernatant was then incubated with 1 ml of anti-FLAG M2 Affinity Gel (Sigma A2220) equilibrated with lysis buffer for 1.5–2 h at 4°C. After thorough washing with lysis buffer (100 column volumes), hDna2 was eluted with seven column volumes of TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 100 μ g/ml of FLAG peptide (Sigma). The amounts of protein recovered varied in each preparation. The hDna2-containing fractions were then loaded onto a 1 ml FPLC MonoQ column equilibrated with MonoQ buffer [150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol (v/v), and 0.1 mM PMSF]. After washing, hDna2 was eluted with a 10 ml gradient of 100–600 mM NaCl in MonoQ buffer. hDna2 was eluted at salt concentrations of 300–350 mM. hDna2-containing fractions were then dialyzed against stock solution [500 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25% glycerol (v/v), 1 mM DTT, 0.15 μ g/ml Leupeptin and 0.1 mM PMSF] and stored at -80°C. Dna2 activity was identified at each purification step by nuclease assay (wild-type and K671E mutant) or ATPase assay (D294A mutant). If necessary, enzymes are further diluted to appropriate concentrations just prior to use in buffer containing 500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 0.5 mg/ml BSA, 10% glycerol (v/v) and 0.02% NP-40.

ATPase assay

Purified hDna2 was incubated in 20 μ l of reaction buffer containing 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM NaCl, 2.5 mM DTT, 0.1 mg/ml BSA, 5% glycerol (v/v), 1 μ g of oligonucleotide (primer, 22 bases) and various concentrations of [γ -³²P]ATP at 37°C. These conditions were determined as optimal in separate titrations of ATP, NaCl and time. The reaction was stopped by adding EDTA to a final concentration of 4 mM, and the reaction mix (0.5 μ l) was spotted onto a polyethyleneimine cellulose plate (SELECTO SCIENTIFIC), which was then developed in 0.5 M LiCl, 1 M formic acid solution. The results were analyzed using the STORM PhosphoImager.

Nuclease assay

In general, nuclease activities of hDna2 were measured using a standard reaction mixture (20 μ l) containing 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2 mM DTT, 0.25 mg/ml BSA, the 5'- or 3'-³²P-labeled DNA substrate, and various concentrations of MgCl₂ and ATP as indicated in the figure legends. NaCl inhibited the nuclease activity (20 times

inhibition at 125 mM) but 25 mM NaCl was minimally inhibitory and included in the reactions to stabilize the oligonucleotide substrates. After incubation at 37°C for 15 min, reactions were stopped with 2× denaturing termination dye (95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), and boiled for 5 min. The cleavage products were separated on a 12% sequencing gel (SequaGel, National Diagnostics) using Model S2 electrophoresis apparatus (BRL, 39 cm plate) and analyzed using the PhosphoImager. Products were quantified using the ImageQuant software on the phosphorimager. Substrate cleaved (%) is calculated as follows: $\text{Substrate cleaved (\%)} = (\text{product bands}) / (\text{substrate bands} + \text{product bands}) \times 100$.

Helicase assay

Helicase assays were performed with the nuclease-deficient mutant of hDna2 (D294A). The standard reaction mixtures contained 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2 mM DTT, 0.25 mg/ml BSA, 4 mM MgCl₂, 4 mM ATP and ³²P-labeled helicase substrate. After incubation at 37°C for 1 h, reactions were stopped with 5× stop solution (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue and 0.25% xylene cyanole FF). Reaction products were then separated using 8% native polyacrylamide gels containing 0.1% SDS, and detected with PhosphoImager.

hDna2 nuclease substrates

All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Oligonucleotide sequences are listed in Table 1. The locations of biotinylation are indicated as underlined nucleotides in the table. The 5' and 3' end labeling of oligonucleotides were performed as described previously (33). Oligonucleotides were annealed as described in the figure legends to form various structures. Flap substrate for the 5'–3' nuclease assay were made by annealing a downstream oligonucleotide, template, and upstream oligonucleotide at molar ratio of 1:2:4. The upstream oligonucleotide was omitted to make the forked substrate.

Flap substrates for 3'–5' nuclease assays were made by annealing a downstream, template, and upstream oligonucleotide at molar ratio of 2:1:2 (Figure 5B and C). To check the specificity of 3'–5' nuclease, flap substrates were made by annealing a downstream template, and upstream oligonucleotide at molar ratio of 4:1:4 (Figure 5A). Upstream oligonucleotides were omitted to make forked substrate.

Both upstream and downstream oligonucleotide were omitted to make single-stranded DNA substrate. For annealing, oligonucleotides were placed in TE (1 mM EDTA and 10 mM Tris-HCl, pH 8.0), heated to 100°C for 5 min, and slowly cooled to room temperature.

hDna2 helicase substrates

The substrate for helicase assay is a 42 base oligonucleotide (HPR) annealed to M13 DNA over 24 bases and having an 18 nt 5' noncomplementary tail (for sequence see Table 1). The oligonucleotide was 5' radiolabeled with [γ -³²P]ATP by T4 polynucleotide kinase in accordance with the manufacturers instructions, annealed to M13 single-stranded DNA (NEB), and purified with a Sepharose CL4B column (Sigma).

Production of anti-human Dna2 antibodies

The plasmid pBS-SK-hDNA2 that contained cDNA encoding hDNA2 (accession no. KIAA0083, KAZUSA DNA Res. Inst.) was used to amplify the N-terminal DNA fragment encoding amino acids 18–353 of hDna2 and a 6× HIS tag at the C-terminus using the primers 5'-GTCCCATATGGAG-CAGCTGAACGAAC-3' and 5'-CAGTCTCGAGGTAGA-GAAGCAAGCC-3'. The amplified fragments were digested with NdeI and XhoI, and cloned into pET-24a(+) vector (Novagen) cleaved with the same restriction enzymes to generate pET-24a(+)-hDna2-NT-HIS. Protein expression and purification was performed by C&P Biotech Corp. (Ontario, Canada). In brief, the protein was expressed in five liters of *Escherichia coli* BL21 (DE3) cells using 0.5 mM Isopropyl- β -D-thiogalactopyranoside at 37°C for 3 h. Cell pellet was harvested, resuspended with lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM benzamidine, 1 μ g/ml of Leupeptin and aprotinin), and disrupted with sonication. Since most of the hDna2-NT-HIS protein was found in inclusion bodies, hDna2-NT-HIS was then solubilized with 8 M urea and 10 mM DTT, and purified with a HisTrap (Pharmacia) column according to manufacturers instructions. Eluted samples were then dialyzed into 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 4 M urea and 1 mM DTT, loaded onto QFF ion-exchange column, and eluted with 0–500 mM NaCl gradient. Fractions from QFF columns were combined, concentrated, and purified further with S200 size exclusion column using 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 M urea and 1 mM DTT.

Anti-Dna2 antibodies were produced against the purified hDna2-NT-HIS protein in rabbits at COVANCE (Denver,

Table 1. Oligonucleotide sequence (5'–3')

Downstream	
D(55mer)	AGGTCTCGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG
D-bio(55mer)	<u>AGGTCTCGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG</u>
Template	
T(51mer)	GCAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGTCCG
T-bio(50mer)	GCAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGT <u>C</u>
Upstream	
U(26mer)	CGACCGTGCCAGCCTAAATTTCAATA
Helicase	
HPR(42mer)	AGCTAGCTCTTGATCGTAGACGTTGTAACGACGGCCAGTG

The underlined nucleotides indicate a biotin modification.

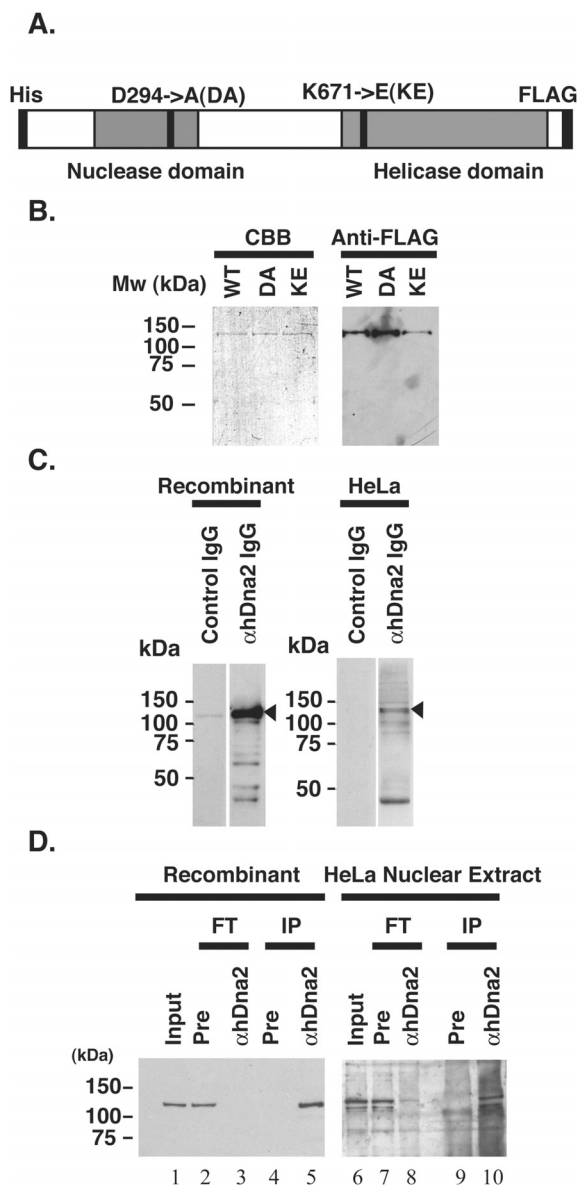


Figure 1. Production of recombinant hDna2 proteins. (A) Schematic diagram of the recombinant human Dna2 proteins. Positions of hDna2 point mutations at conserved amino acids of the nuclease domain (D294A) and the helicase domain (K671E) are shown. (B) Purified recombinant hDna2 proteins. Recombinant hDna2 wild type (WT), nuclease negative (DA) and ATPase negative (KE) were subjected to SDS-PAGE and stained with Coomassie staining (CBB) or detected with anti-Flag antibodies (Anti-FLAG). Molecular weight markers are shown on the left. (C) Detection of hDna2 in human cells. Purified hDna2 (Recombinant) or HeLa nuclear extract (HeLa) were separated by SDS-PAGE, and detected with western blotting using either affinity purified polyclonal antibody against N-terminal amino acids of hDna2 (α hDna2 IgG) or control antibodies (Control IgG). Positions of hDna2 are indicated by arrowhead. (D) Immunoprecipitation of hDna2. Recombinant hDna2 (lanes 1–5) or HeLa nuclear extract (lanes 6–10) were immunoprecipitated with pre-immune serum (Pre) or anti-hDna2 (α hDna2) antibodies, and detected with western blotting using anti-hDna2-NT antibodies. Lanes 1 and 6, input; lanes 4, 5, 9 and 10, immunoprecipitates; lanes 2, 3, 7 and 8, supernatant of the immunoprecipitation.

PA). Anti-Dna2 antibodies were purified by affinity chromatography (34) on Affigel 15 (BioRad) containing covalently bound hDna2-NT-HIS. Control IgG was purified from pre-immune serum using protein A beads (Amersham).

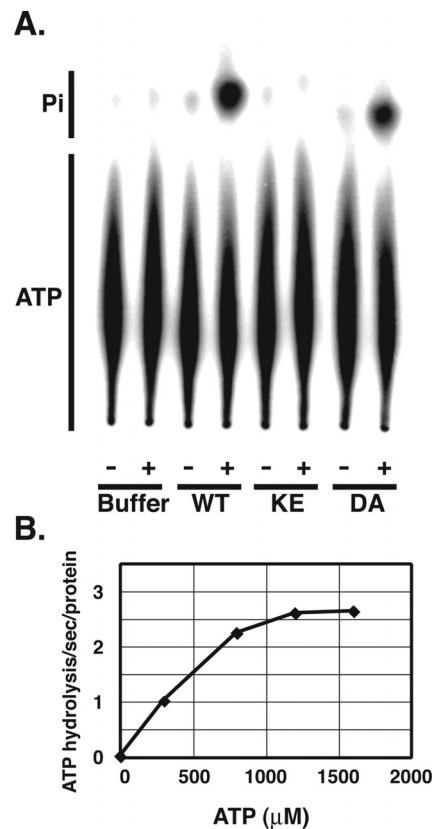


Figure 2. ATPase activity of recombinant hDna2 proteins. (A) DNA-dependent ATPase activity of human DNA2. ATPase activity of recombinant hDna2 wild type (WT), D294A (DA) or K671E (KE) were measured using reaction mixtures containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence (+) and absence (–) of single-stranded DNA as described in Materials And Methods. Reactions contained 600 fmol of recombinant protein and 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After incubation for 20 min at 37°C, the reaction products were separated by thin layer chromatography and detected using PhosphoImager. ATP indicates the position of uncleaved ATP. Pi indicates position of released inorganic phosphate. As a control, protein was omitted from the reaction (Buffer). (B) Extent of ATPase. The ATPase activity of wild-type hDna2 was measured using reaction mixtures containing various concentrations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and extents of ATP hydrolysis were plotted against ATP concentration. To normalize background, ATPase activity was measured in the absence of single-stranded DNA and subtracted.

Immunoprecipitation of hDna2 proteins

HeLa cell nuclear extracts were the gift of Carl Parker (California Institute of Technology, Pasadena, CA). 20 μ l of protein A beads (Amersham) was incubated with 10 μ l of anti-Dna2 serum or control serum for 30 min at 4°C, and then washed with IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 15 mM DTT and 0.2% NP-40). HeLa nuclear extract (25 μ l) or recombinant hDna2 (1.2 μ g) was then incubated with beads from above for 1 h at 4°C. After incubation, the beads were washed with IP buffer, and immunoprecipitates were analyzed by western blotting. The supernatants (10 μ l) of the immunoprecipitation were also assayed.

Preparation of experimental data as computer images

Images were edited using the levels command of Adobe Photoshop. Specifically, images were enhanced by dragging

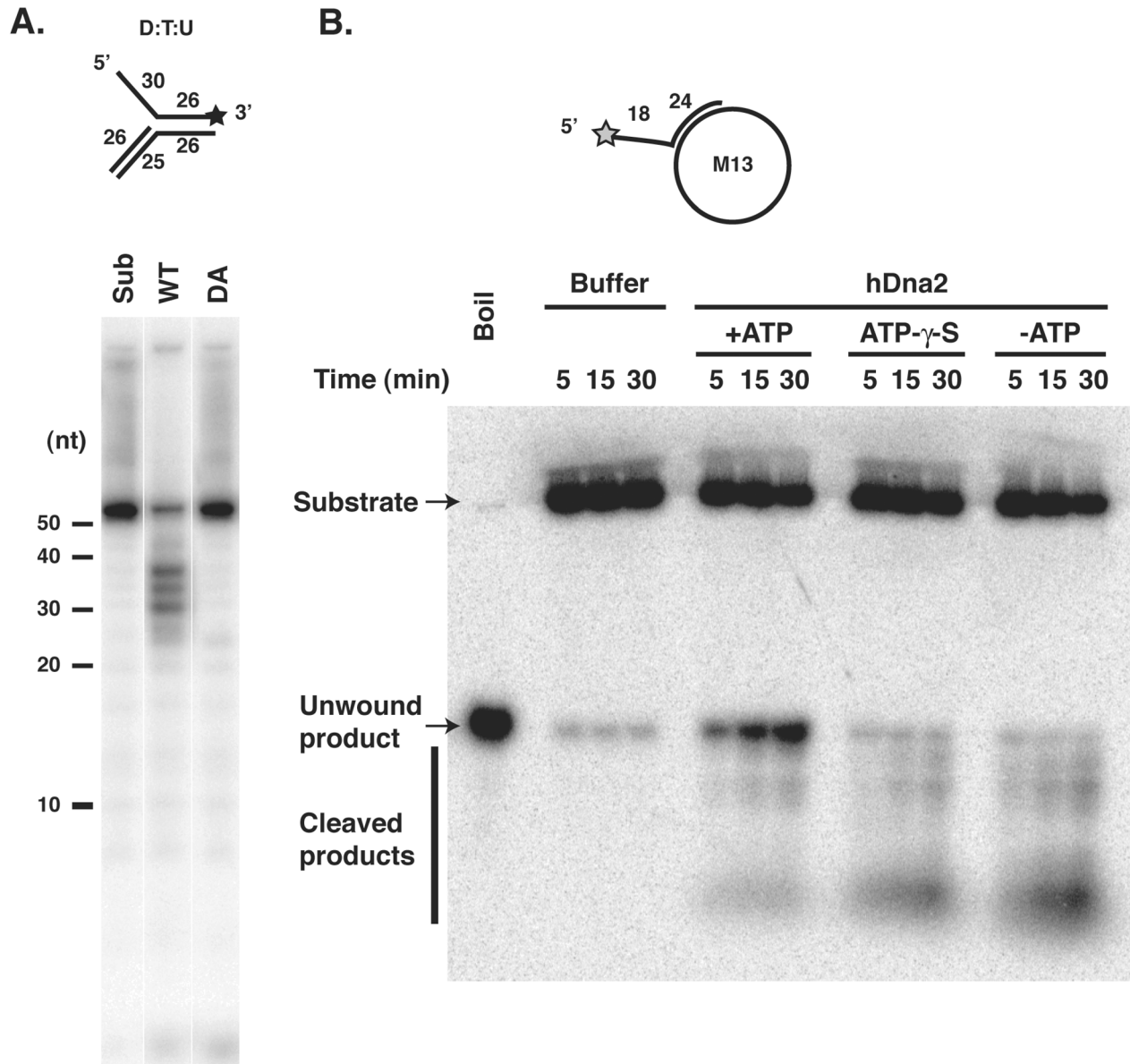


Figure 3. Helicase activity of recombinant hDna2 proteins. (A) Effect of D294A mutation on the 5'–3' nuclease activity of hDna2. The 5'–3' nuclease activity of hDna2 was measured as described in Materials And Methods. Reaction mixtures contained either hDna2 wild type (WT) or D294A mutant (DA) (300 fmol). As a negative control, enzyme was omitted from the reaction (Sub). The flap (D:T:U) substrate used (15 fmol) is shown at the top of the figure (star, ³²P-labeled ends). Numbers shown on the left of the figure indicate the size of the markers. Proteins were preincubated with substrates before adding Mg⁺⁺ to a final concentration of 0.5 mM, and then further incubated for 10 min at 37°C. (B) Helicase activity of recombinant hDna2. The 5' end-labeled oligonucleotide annealed with M13 single-stranded DNA (star, ³²P-labeled end) was incubated with hDna2D294A in the presence of ATP (+ATP), or ATP-γ-S (ATP-γ-S) as indicated. As a control, ATP was omitted from the reaction (–ATP). Reactions were stopped at indicated times by adding 5× stop solution. Products were then separated using native gel electrophoresis and detected by autoradiography. Boil denotes boiled substrate to indicate the position of the unwound strand. As a control, hDna2 was omitted from the reaction mixture (Buffer). Positions of substrate (Substrate), helicase products (Unwound product) and nuclease products (Cleaved products) are as indicated on the left.

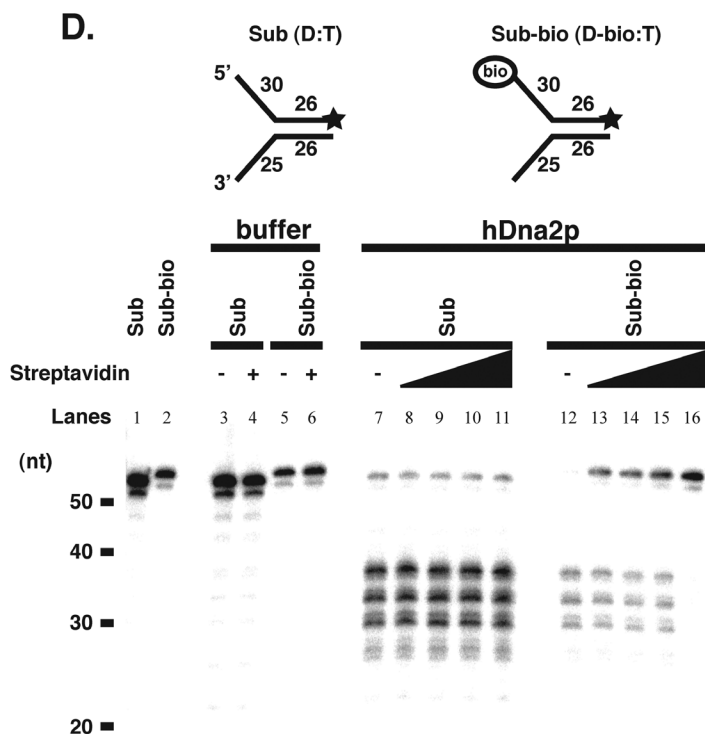
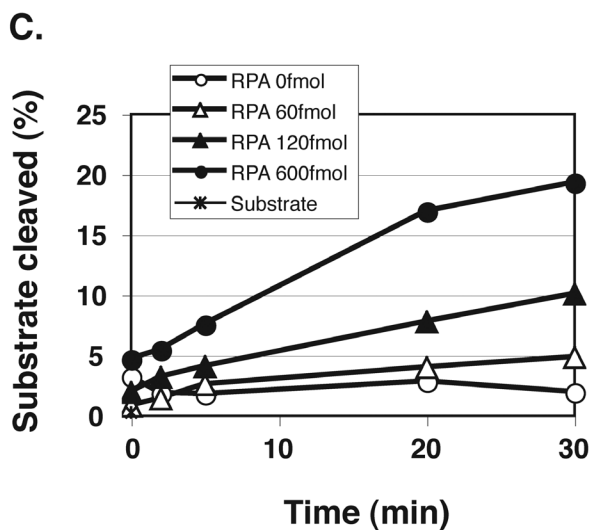
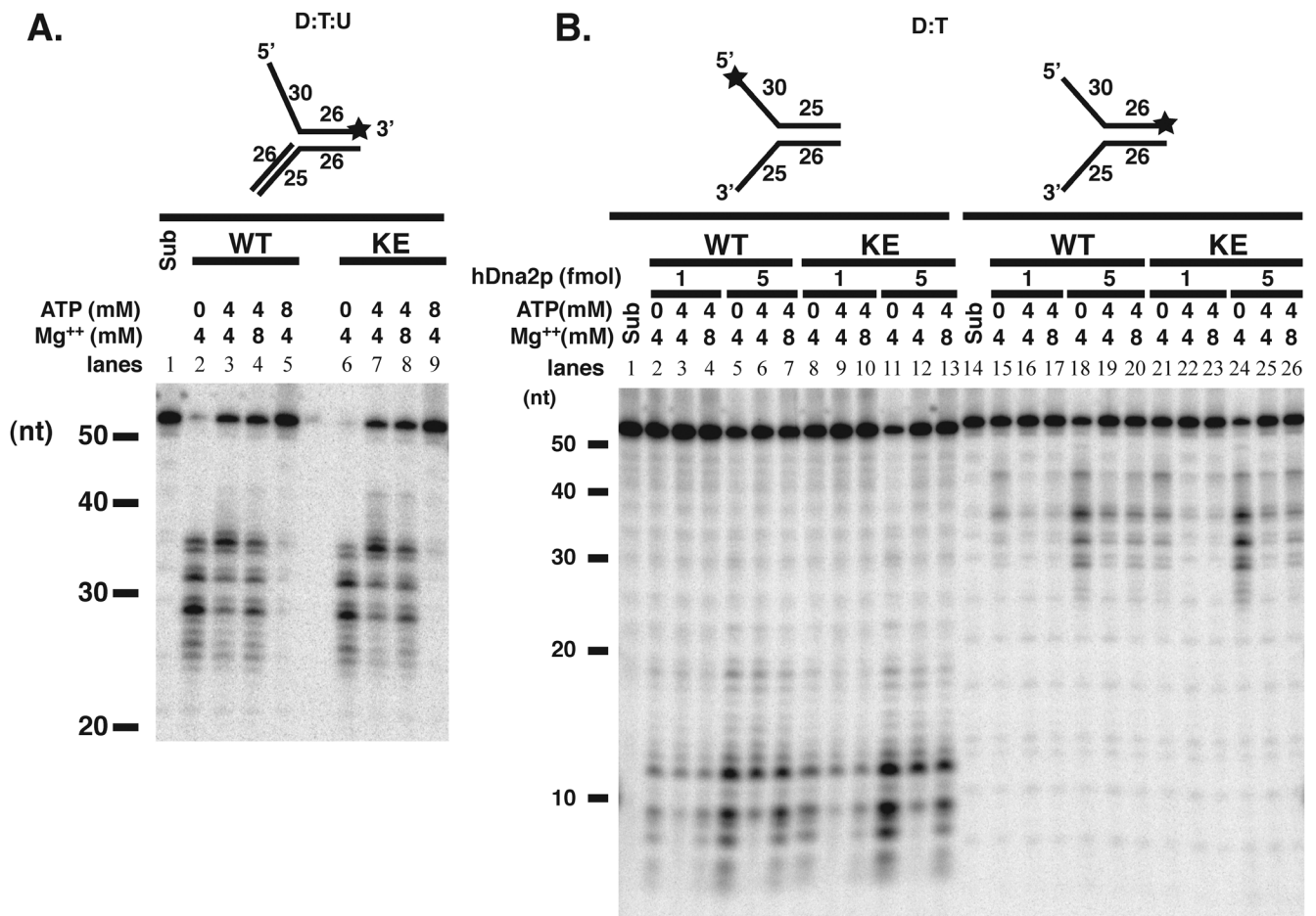
the black and white input levels sliders to the edge of the first group of pixels on either end of the histogram.

RESULTS

Preparation of recombinant human Dna2 protein from baculovirus-infected insect cells

Previously, we have shown that DNA2L, cDNA accession number KIAA0083 (Swiss prot accession no. P51530,

Genbank accession no. D42046), can complement the yeast *dna2-1* mutant (30). This result strongly suggests that DNA2L is the human ortholog of ScDNA2, although the enzymatic properties of hDna2 protein have not been investigated. To learn if the hDna2 has similar biochemical activity to that of ScDna2, we purified recombinant hDna2. The human *DNA2* gene was tagged at the N-terminus with six histidines and at the C-terminus with a FLAG tag, cloned into a baculovirus expression vector, expressed in High5 insect cells, and purified as described under Materials And Methods. To prepare



nuclease-deficient and ATPase-deficient mutant enzymes, conserved amino acids in hDna2 corresponding to those critical for endonuclease and ATPase of budding ScDna2 were changed (Figure 1A) (6,7). Purification of recombinant hDna2 was confirmed using anti-FLAG antibodies (Figure 1B, right). Purity of the recombinant proteins was estimated as >90% on Coomassie-stained gels. Similar levels of purity were obtained with each of the mutant proteins (Figure 1B, left).

We then performed western blotting to establish the presence of endogenous hDna2 in human cells. Recombinant hDna2 or HeLa nuclear extract were separated by SDS-PAGE, and hDna2 was detected after blotting using polyclonal antibody raised against hDna2 N-terminal fragment. Anti-hDna2 antibody detected a protein of ~120 kDa from both recombinant hDna2 and human nuclear extract (Figure 1C). Since the molecular weight of hDna2 expected from its amino acid sequence is 119 kDa, this result strongly suggests the cDNA clone encodes full-length hDna2 in human nuclei. The anti-hDna2 antibody could immunoprecipitate a protein of 120 kDa both from a solution of recombinant hDna2 and from HeLa nuclear extracts, further supporting our conclusion that this band corresponds to hDna2 (Figure 1D).

DNA-dependent helicase and ATPase activities of recombinant hDna2 proteins

ScDna2 possesses single-stranded DNA-dependent ATPase activity (5,6). To see if the enzymatic activity of ScDna2 is conserved in human, we investigated the ATPase and helicase activity of recombinant hDna2. As shown in Figure 2, wild-type hDna2 showed ATPase activity, optimized as described in Materials And Methods. The ATPase activity of hDna2 required single-stranded DNA, indicating a similar cofactor requirement to that of ScDna2. The ATPase activity of hDna2 proteins were calculated at a time point that gives initial rate of ATP hydrolysis, and the rate of ATP hydrolysis was 10% of ScDna2 (Figure 2B, Discussion). The apparent K_m for the ATPase activity was 400 μM . The K671E mutation (KE) abolishes the ATPase activity, confirming that the Walker A box is part of the active site of the DNA-dependent ATPase. On the other hand, a mutation in the nuclease domain (D294A, DA) had no effect on the ATPase activity (Figure 2).

ScDna2 possesses a distributive helicase activity, and unwinds a DNA duplex with a 5' tail in an ATP hydrolysis dependent manner (5,12,13). Therefore, we next investigated

the helicase activity of recombinant hDna2. For this assay, a mutant hDna2 with a point mutation in a conserved amino acid in the nuclease domain (hDna2D294A) was used to prevent degradation of substrate. The D294A mutation abolished most of the nuclease activity of hDna2, as expected (Figure 3A). As a substrate for measuring unwinding, we used an oligonucleotide annealed to M13 single-stranded phage DNA, which yields a forked structure with a noncomplementary 5' tail of 18 nt. This kind of molecule has been shown previously to be a good substrate for the DNA helicase activity of ScDna2 (5,6). As shown in Figure 3B, hDna2D294A showed significant displacement of the labeled oligonucleotide (Figure 3B, Unwound product). The unwinding is dependent on ATP, and ATP- γ -S will not substitute, indicating that unwinding is dependent on ATP hydrolysis. We detect some residual nuclease activity in the absence ATP, but addition of ATP significantly suppressed this nuclease activity (Figure 3B, Cleaved products). Since strand displacement is dependent on ATP hydrolysis and residual nuclease activity is inhibited by ATP, we conclude that the displaced strand is not a product of nuclease activity, but a product of helicase activity. These results indicate that recombinant hDna2 possesses DNA helicase activity.

Analysis of the 5'-3' nuclease activity of hDna2

It has previously been shown that the 5'-3' nuclease activity of ScDna2 is essential (2,7). ScDna2 cleaves forked or flap substrates adjacent to the duplex region, resulting in products appropriate for further processing by FEN1 nuclease. The nuclease activity of ScDna2 is regulated by ATP. Addition of ATP suppresses the nuclease activity and activates DNA helicase activity, allowing unwinding of flap substrates and cleavages beyond flap base (5,7,12,13). To see if these properties of ScDna2 are conserved in hDna2, we next investigated the spectrum of hDna2 nuclease products in the presence of various concentrations of ATP. To accomplish this, we employed flap or forked substrates labeled at either the 3' or 5' end of the downstream oligonucleotide (Figure 4A and B). Figure 4A shows that the final products of nuclease (labeled at the 3' end) are around 25–35 nt long, which means that hDna2 cleaves the flap or fork up to a few nucleotides from the base of the flap (lane 2). This cleavage pattern is similar to that of ScDna2 in the absence of ATP (35). Addition of ATP reduced the extent of the nuclease activity (lane 3). Nuclease products with length of <25 nt were not observed,

Figure 4. The 5'-3' nuclease activity of recombinant hDna2. (A) Effect of ATP on the 5'-3' nuclease activity of hDna2. The 5'-3' nuclease activity of hDna2 was measured in the presence of various concentrations of ATP and Mg^{++} as described in Materials And Methods. The concentrations of ATP and Mg^{++} are as shown on the figure. Reaction mixtures contained either hDna2 wild type (WT, lanes 2–5) or ATPase negative (KE, lanes 6–9) (20 fmol). As a negative control, enzyme was omitted from the reaction (Sub, lane 1). The flap substrate used (D:T:U, 15 fmol) is shown at the top of the figure. Numbers shown on the left of the figure indicate the size of the markers. Proteins were preincubated with substrates for 5 min at 4°C before adding Mg^{++} . (B) Determination of cutting position of 5'-3' nuclease activity of hDna2. Nuclease assays were performed as described in (A) using 5' (lanes 1–13) or 3' (lanes 14–26) end-labeled forked substrates (D:T) without preincubation. Reaction mixtures contained either hDna2 wild type (WT, lanes 2–7 and 15–20) or ATPase negative (KE, lanes 8–13 and 21–26). 1 fmol (lanes 2–4, 8–10, 15–17 and 21–23) or 5 fmol (lanes 5–7, 11–13, 18–20 and 24–26) of hDna2 wild type are used as shown in the figure. (C) Effect of RPA on the 5'-3' nuclease activity of hDna2. Time course of 5'-3' nuclease activity of hDna2 (30 fmol) on flap substrate (D:T:U, 30 fmol + 30 fmol T + 90 fmol U) was assayed in the presence of increasing amounts of hRPA (0–600 fmol) as shown in the figure. NaCl (125 mM) was included to suppress destabilization of the substrate by hRPA. Reaction mixtures were preincubated without hDna2 at 4°C for 10 min to allow binding of RPA to the substrates. Percentage substrate cleaved was determined as described in Materials And Methods. (D) The 5'-3' nuclease activity of hDna2 requires a free 5' end. The 5'-3' nuclease activity of wild-type hDna2 (20 fmol) was measured using unmodified [D:T (Sub), lanes 1, 3–4 and 7–11] or 5' biotinylated [(D-bio:T (Sub-bio), lanes 2, 5–6 and 12–16] fork substrates (15 fmol) in the presence of increasing amounts (0, 2.5, 5, 10 and 20 fmol, as indicated by triangles) of streptavidin. Reaction mixtures were preincubated at 4°C for 15 min without enzymes to promote formation of biotin-streptavidin complex. As negative control, unbiotinylated (lanes 3 and 4) or biotinylated (lanes 5 and 6) substrates were incubated in the absence (–, lanes 3 and 5) or presence (+, 20 fmol, lanes 4 and 6) of streptavidin without hDna2. Lanes 1 and 2 show substrate only control.

indicating that cleavage products beyond the flap base were not produced for hDna2 even in the ATP/Mg⁺⁺ ratio (1:1), which allows unwinding of 5'-tailed partial duplex DNA (Figure 3). Surprisingly, the KE mutation that abolishes ATPase activity did not affect the suppression of nuclease activity by ATP (Figure 4A, compare WT with KE). This result may indicate that the change of cleavage position in the presence of ATP is not dependent on binding of ATP to the Walker A box, although this mutation has not been shown directly to abolish ATP binding in Dna2. Nor did the addition of excess Mg⁺⁺ over ATP prevent suppression of the nuclease by ATP (lanes 3, 4, 7 and 8); thus the suppression is not likely due to titration of Mg⁺⁺.

We next investigated the nuclease activity using a forked substrate labeled at the 5' end to investigate sequential cleavage from the end of strand (Figure 4B, left). This substrate produced nuclease products of >5 nt, suggesting that the first cleavage position by hDna2 is around 5 nt away from the 5' end (lanes 2, 5, 8 and 11). Addition of ATP resulted in the release of longer nuclease products, again indicating an inhibition of nuclease by ATP, although the KE mutation did not affect the inhibition (Figure 4B, lanes 3, 4, 6, 7, 9, 10, 12 and 13). We could not detect cleavage in the duplex DNA region (>30 nt), however, consistent with the results using 3' end-labeled substrate (Figure 4B, compare lanes 2–7 and 15–20). These results show that cleavage points of flap and fork substrates by the 5'–3' nuclease of hDna2 are very similar to those of ScDna2, although hDna2 cannot cleave the double-stranded region beyond the flap base, even in the presence of ATP.

It has been shown that the enzymatic activities of ScDna2 can be stimulated by yeast RPA, indicating a functional interaction between these two proteins (36). To determine if the interaction is conserved in human proteins, we next assayed stimulation of hDna2 by human RPA (hRPA). RPA itself destabilized the duplex DNA substrates at the low salt concentrations optimal for Dna2 activities (25 mM and below). We found that the duplex can be stabilized in the presence of RPA, however, by the addition of NaCl to the reaction. NaCl was titrated into reaction mixtures containing RPA to determine a minimal concentration where the duplex was stable even in the presence of 4-fold excess of RPA and the nuclease was still active. This NaCl concentration was found to be 125 mM. As with yeast Dna2 [(37) and data not shown], 125 mM inhibited hDna2 about 20-fold (as determined by quantitative comparison of data from Figure 4A and B with Figure 4C). Figure 4C shows a time course of nuclease activity in the presence of increasing amounts of hRPA. Addition of RPA stimulated the nuclease activity up to 8- to 10-fold, in a dose-dependent manner. These results indicate that the 5'–3' nuclease activity of hDna2 is stimulated by hRPA.

Yeast Dna2 prefers free 5' ends, and conjugation of biotin–streptavidin at the 5' end can suppress 5'–3' nuclease activity (18). To see if steric hindrance at the 5' end inhibited the 5'–3' nuclease activity of hDna2, we next placed a biotin modification at the 5' end of the flap substrate and investigated effects of a biotin–streptavidin complex on nuclease cleavage. As shown in Figure 4D, cleavage of biotinylated substrate was inhibited by increasing amounts of streptavidin, although unmodified forks were cleaved equally well in the absence or presence of streptavidin (compare lanes 7–11 and 12–16).

This result indicates that the 5'–3' nuclease activity of hDna2 requires a free 5' end and that hDna2 may therefore use the same tracking mechanism as ScDna2.

3'–5' Nuclease activity of hDna2

It has been reported that ScDna2 has 3'–5' nuclease activity in addition to 5'–3' nuclease (35). To learn more about the similarity of yeast and human Dna2, we next investigated the 3'–5' nuclease activity of hDna2. We first compared nuclease products of forked and flap substrates. As shown in Figure 5A, hDna2 showed 3'–5' nuclease activity against single-stranded DNA and a forked substrate, but not against a 5'-flap substrate with fully duplex 3' ends. This result indicates that hDna2 has 3'–5' nuclease activity specific to single-stranded ends. The length of the nuclease product from a forked substrate labeled at the 5' end was around 35 nt, indicating that around 10 nt of single-stranded DNA at the fork was left uncleaved after the reaction (lanes 9–11). This spectrum of cleavage products differs from that of the 5'–3' nuclease, which can cleave closer to the duplex region (Figure 4). Like, the 5'–3' nuclease, the 3'–5' nuclease activity was suppressed by the D294A mutation, showing that the same active site is employed for both 5'–3' and 3'–5' nuclease activities (Figure 5A, DA).

To see if the 3'–5' nuclease activity of hDna2, like the 5'–3' activity, also requires free ends, we next placed biotin at the 3' end of the substrate and investigated the effect of streptavidin on the nuclease activity of hDna2. As shown in Figure 5B, cleavage of biotinylated substrate was inhibited by increasing amounts of streptavidin, although substrates lacking biotin were cleaved equally well in the absence or presence of streptavidin (compare lanes 7–11 with 12–16). This result indicates that the 3'–5' nuclease activity of hDna2 requires a free 3' end, and may employ a similar tracking mechanism to that of the 5'–3' nuclease, consistent with there being a single active site for both nuclease activities.

Finally, we investigated effects of ATP on the 3'–5' nuclease activity using various concentrations of ATP and Mg⁺⁺ as shown in Figure 5C. In the absence of ATP, the length of nuclease products was mainly 30–35 nt. In the presence of ATP, nuclease activity was reduced and released slightly longer nuclease products (30–40 nt) indicating inhibition of nuclease (lanes 3 and 4). However, cleavage products below 30 nt were not detected in the presence of ATP, again indicating that there is no cleavage in the double-stranded region. This result is consistent with the behavior of the 5'–3' nuclease, and indicates that there is no unwinding of the fork substrate before cleavage.

DISCUSSION

In this paper, we investigated the enzymatic activities of hDna2 to obtain insights into the function of Dna2 in human cells. Our results show that hDna2 is a helicase/nuclease with similar properties to those of Dna2 orthologs in other organisms, indicating the functional conservation of Dna2.

ScDna2 possesses a distributive helicase/ATPase activity, and can unwind DNA duplex with 5'-tail (5,6,12,13). Here, we showed that recombinant hDna2 also has ATPase/helicase

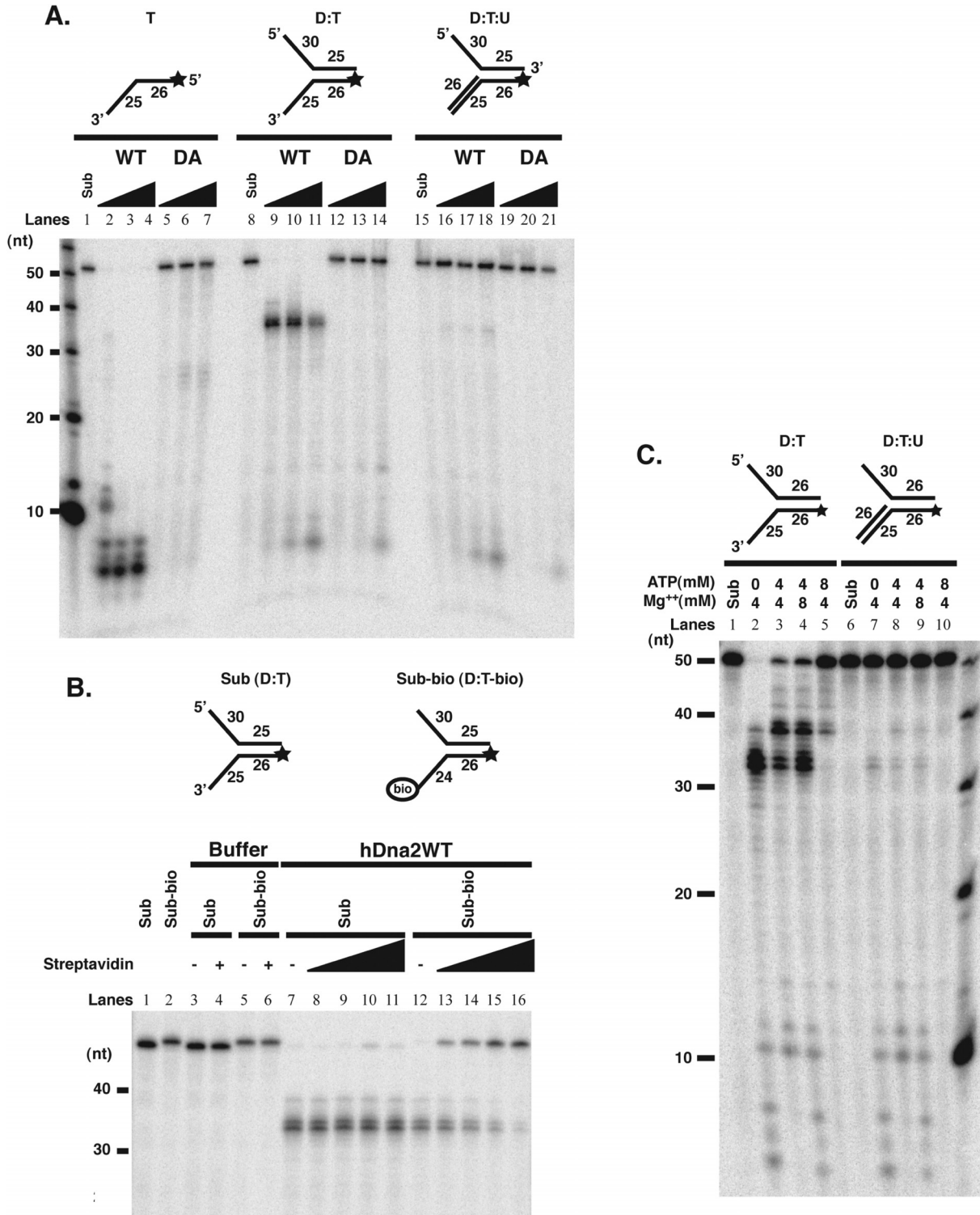


Figure 5. The 3'-5' nuclease activity of hDna2. (A) The 3'-5' nuclease activity of hDna2 is specific to single-stranded region. 3'-5' nuclease activity of hDna2 was measured with 15 fmol of single-stranded DNA (T, lanes 1-7), forked substrate (D:T, lanes 8-14), or flap substrate (D:T:U, lanes 15-21) in the presence of increasing amounts (20, 100 and 600 fmol, as indicated by the triangles) of hDna2 wild type (WT, lanes 2-4, 9-11 and 16-18) or nuclease-defective (DA, lanes 5-7, 12-14 and 19-21). Enzyme was omitted from the reaction as negative control (Sub, lanes 1, 8 and 15). Reaction mixture contained 4 mM Mg⁺⁺ and no ATP. Proteins were preincubated with substrate for 5 min at 4°C before adding Mg⁺⁺. (B) The 3' nuclease activity of hDna2 requires a free 3' end. Effect of increasing amounts of streptavidin (0, 2.5, 5, 10 and 20 fmol, as indicated by triangles) on 3'-5' nuclease activity of wild-type hDna2 (20 fmol) was investigated using unmodified [D:T, (Sub), lanes 1, 3-4 and 7-11] or 3' biotinylated fork [D:T-bio (Sub-bio), lanes 2, 5-6 and 12-16] substrates (15 fmol) as described in Figure 4D. (C) The 3'-5' nuclease activity of hDna2 is inhibited by ATP. The 3'-nuclease activity of hDna2 wild type (20 fmol) was measured in the presence of various concentrations of ATP and Mg⁺⁺ as described in Figure 4A. The forked (D:T, lanes 1-5) and flap (D:T:U, Lanes 6-10) substrates used (15 fmol) are shown at the top of the figure. As a negative control, enzyme was omitted from the reaction (Sub, lanes 1 and 6).

activity (Figures 2 and 3). This activity is intrinsic to the recombinant hDna2, because mutation of the ATPase domain resulted in the inactivation of ATPase. The rate of ATP hydrolysis was quite low, 2.6/s, around 10% of the ATP hydrolysis rate reported for ScDna2 (Figure 2) (7). Consistent with the low ATPase activity, the helicase activity of hDna2 is not high. We needed excess hDna2D294A to detect displacement of substrate (Figure 3), and cleavages by the nuclease beyond the flap base, which have been reported for ScDna2, were not observed for hDna2, even at the ATP/Mg⁺⁺ ratio that allows nuclease-defective hDna2 to unwind a 5'-tailed, partial duplex DNA (12,13) (Figures 4 and 5). One possible explanation for this low ATPase/helicase activity of hDna2 is that substrates used in this experiment are not the endogenous substrate of hDna2. For example, ScDna2 shows dynamic localization to telomeres, and is involved in telomerase-dependent and telomerase-independent telomere elongation, suggesting that Dna2 acts on telomere DNA (3). Alternatively, our previous results using substrates with secondary structure showed that the helicase activity of ScDna2 is essential for effective nuclease cleavage of this substrate (16). The helicase activity of Dna2 may be specialized for processing of these special sequences.

Previous reports have shown that the nuclease active site of ScDna2 is required for the viability of budding yeast, and it was proposed that the function of Dna2 is processing of 5'-flap structures in cooperation with RPA and FEN1 during Okazaki fragment maturation (2,7,36). We investigated the 5'-3' nuclease activity of the hDna2 to see if hDna2 has similar nuclease activity to that of ScDna2. As shown in Figure 4, the position of the cutting in the absence of ATP is near the base of the displaced single strand, indicating a striking similarity of the nuclease specificity between human and yeast (35). Other features that suggest similarity between the nuclease activities of ScDna2 and hDna2 include (i) cleavage position near the end of strand (Figure 4B) (12), (ii) suppression of nuclease activity by steric hindrance from biotin-streptavidin conjugation at 5' end (Figure 4C) (18), (iii) activation of 5'-3' nuclease by hRPA (Figure 4D) (36), and (iv) Inhibition of nuclease by ATP (5,7,9,14). One difference is apparent lack of coupling of nuclease and helicase in hDna2. The coupling of nuclease and helicase may be important when Dna2 processes flap substrates with secondary structures (16). However, we could not detect nuclease product cleaved in the double-stranded region of flap substrates, perhaps because addition of ATP resulted in the inhibition of nuclease (Figure 4A and B). The helicase activity of hDna2 may be too weak to allow the coupling of nuclease and helicase, or a special substrate is required to show optimum helicase activity (see above). Regulation of nuclease activity by ATP is interesting, since it is conserved between yeast and human Dna2. ATP may titrate Mg⁺⁺ or may cause a change of Dna2 structure, leading to the suppression of nuclease.

It has been shown that ScDna2 possesses 3'-5' nuclease activity in addition to 5'-3' nuclease (6,35), but the activity was lower than that of the 5'-3' nuclease, and the function of the nuclease has not been established. In this paper, we performed detailed analysis of the 3'-5' nuclease activity of hDna2. As shown in Figure 5, hDna2 also showed 3'-5' nuclease activity, indicating that this is a conserved activity of Dna2 orthologs. This activity is specific for a

single-stranded region, is inhibited in the presence of ATP, and needs a free 3' end of single-stranded DNA for activity. The requirement for a free 3' end suggests that the 3'-5' nuclease employs a similar tracking mechanism to that of the 5'-3' nuclease activity (Figures 4D and 5B). The 3'-5' nuclease activity was suppressed by the D294A mutation to the same extent as the 5'-3' nuclease activity, indicating that the same active site is employed for both nuclease activities. On the other hand, regulation of these two nuclease activities is not exactly the same, since hRPA did not stimulate 3'-5' nuclease activity (data not shown), and the spectrum of cleavage positions is different (compare Figures 4A and 5A). This kind of dual-polarity nuclease activity has been reported in *E.coli* RecB enzyme. As in hDna2, the same active site in RecB catalyzes both nuclease activities, with the 3'-5' activity differentially inhibited by the presence of the specific chi sequence (38). There might be a similar switch in Dna2 helicase, though further investigation is required to determine the role of the 3'-5' nuclease. This finding leads to the speculation that Dna2 may function on equilibrating flaps at Okazaki fragment junctions in conjunction with DNA polymerase δ . Such a model would account for the synthetic lethality of *dna2-1* and *pol3-01*, a mutant defective in the 3' exonuclease exonuclease of pol δ and may also be related to the suppression of *dna2-1* by *pol32 Δ* , a mutant that may lead to decreased strand displacement by pol δ (8).

In conclusion, we believe this gene is a functional ortholog of ScDNA2 for the following reasons. (i) hDna2 shows conservation throughout the length of the protein coding sequence, including the nuclease domain (29). (ii) The enzymatic properties of hDna2 are strikingly similar to those of ScDna2. (iii) The hDNA2 gene can complement temperature-sensitive phenotype of Sc *dna2-1* (30). We are now planning further studies, using the specific antibodies described here, of the *in vivo* function of hDna2 in human cells, which will give us insights into the role of Dna2 in DNA replication, tumorigenesis, telomere maintenance and aging in human cells.

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