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Single Strand Annealing and ATP-independent Strand Exchange Activities of Yeast and Human DNA2

*POSSIBLE ROLE IN OKAZAKI FRAGMENT MATURATION**

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Taro Masuda-Sasa[1], **Piotr Polaczek**[1], **and Judith L. Campbell**[2]

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

The Dna2 protein is a multifunctional enzyme with 5′-3′ DNA helicase, DNA-dependent ATPase, 3′ exo/endonuclease, and 5′ exo/endonuclease. The enzyme is highly specific for structures containing single-stranded flaps adjacent to duplex regions. We report here two novel activities of both the yeast and human Dna2 helicase/nuclease protein: single strand annealing and ATP-independent strand exchange on short duplexes. These activities are independent of ATPase/helicase and nuclease activities in that mutations eliminating either nuclease or ATPase/helicase do not inhibit strand annealing or strand exchange. ATP inhibits strand exchange. A model rationalizing the multiple catalytic functions of Dna2 and leading to its coordination with other enzymes in processing single-stranded flaps during DNA replication and repair is presented.

Yeast Dna2 protein (yDna2)[3] is a helicase/nuclease involved in the maintenance of genome stability (1). A human ortholog of yDna2, hDna2, has recently been characterized and was found to have biochemical features remarkably similar to its yeast counterpart (2, 3). When *DNA2* is deleted from otherwise wild-type yeast cells, cells are inviable, suggesting that *DNA2* is essential. However, the inviability of a *dna2*Δ strain can be suppressed by deletion of *PIF1*, encoding another helicase, and therefore, under this condition, *DNA2* is not essential (4). The helicase activity modulates the preference of Dna2 for substrates with specific structures (5). Recessive mutations in the helicase domain are lethal, suggesting that the helicase is essential under most conditions (6). In some circumstances, however, full helicase activity is not essential for viability (7). Mutations eliminating nuclease activity are lethal but are deleterious even in the presence of wild-type Dna2 (8, 9).

The precise biological role of Dna2 is not clear. The recently described comprehensive genomic integrity network in yeast, derived from a screen for genes synthetically lethal with *dna2,* points to not just one, but to a variety of cellular processes in which Dna2 may function, including DNA replication, DNA repair, and chromatin dynamics (1). The proposed role of Dna2 in Okazaki fragment processing has been studied most extensively (10). As a result of biochemical reconstitution studies one may conclude that Dna2 has an important role in the nucleolytic removal of RNA/DNA primers during the maturation of intermediates in lagging strand DNA synthesis (11, 12). In this scenario, DNA polymerase δ (pol δ) is proposed to displace a 5′ RNA/DNA flap at the terminus of the previously synthesized Okazaki fragment (11). The principal nuclease involved in the removal of the flap in preparation for joining of the fragments is FEN1 (11), the product of the *RAD27* gene. Normally, pol δ and FEN1 activity are strictly coordinated to ensure efficient flap removal (12). However, FEN1 is very inefficient in cleaving long flap structures bound tightly to RPA (11). Such flaps may arise during replication due to excessive strand displacement, which can result either from failure to properly coordinate FEN1 and pol δ, or to other disruptions of the polymerase cycle. Dna2, on the other hand, is well suited to perform the task of long flap cleavage. The helicase activity allows the processing of flaps containing duplex foldbacks that might arise when copying sequences containing repeats (5). The nuclease activity requires long flaps and is stimulated by RPA (5, 11). Therefore, the essential function of *DNA2* may be the processing of long flap structures and/or structured flaps, providing an optimum substrate for FEN1, and thus preventing replication fork arrest (5, 12–16).

While consistent with biochemical reconstitution experiments, this model is also supported by genetic evidence. First, *dna2* is synthetically lethal with *rad27* (10). Overproduction of FEN1 leads to suppression of *dna2-1* lethality. This may suggest that increase in gene dosage of *RAD27* compensates for *DNA2* deficiency, likely caused by either increased processing of the long flaps or to decreased strand displacement by pol δ (10). Second, *rad27 pol3-01*, and *dna2-1 pol3-01* double mutants are both inviable (1, 15). This may suggest that the absence of pol δ 3′ to 5′ exonuclease activity leads to excessive strand displacement and creation of long flap substrates that would require the cooperative action of FEN1 and Dna2 for processing (14). Moreover, lethal interactions are observed, not just between *DNA2* and *RAD27*, but between *DNA2* and most of the key proteins participating in this process (1).

Deletion of *SGS1*, which encodes a 3′-5′ helicase of the RecQ family (17), also involved in maintaining genome stability, is synthetically lethal with *dna2-2* mutations (1, 18). Interpretation of the *dna2-2 sgs1*Δ synthetic lethality is more complex

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Braun Laboratories 147-75, CA Institute of Technology, Pasadena, CA, 91125. Tel.: 626-395-6053; Fax: 626-449-0756; E-mail: jcampbel@its.caltech.edu.

[3] The abbreviations used are: yDna2, yeast Dna2 protein; MOI, multiplicity of infection; nt, nucleotide; HA, hemagglutinin.

than that of *dna2 rad27*Δ. Sgs1 is not known to be involved in Okazaki fragment processing; instead, it is thought to participate in repair of stalled replication forks (19). However, *rad27*Δ *sgs1*Δ double mutants are also inviable (20). Thus, Sgs1 may provide functions in DNA metabolism in the absence of Dna2 other than those documented to date. To identify these functions, we searched for suppressors of *dna2-1* defects among the RecQ homologs. Overproduction of yeast *SGS1* was toxic, even in wild-type strains. However, the human BLM gene, an ortholog of *SGS1*, rescues the *dna2-1* defect (21). We were unsuccessful in showing suppression by the mouse WRN (21), another RecQ family member, but human WRN does suppress *dna2-1* (22). This raised the question as to what activity of human BLM or WRN is responsible for the suppression. Both hBLM and hWRN proteins were shown to stimulate FEN1 cleavage (22–24). Therefore, one possible explanation for the suppression of *dna2-1* is that the increased efficiency of FEN1 cleavage may compensate for the Dna2 defect, correlating well with the *in vivo* results.

Studies in our laboratory have shown that the helicase activity of BLM may be important for the suppression of *dna2-1*. This is puzzling because Dna2 and hBLM helicases differ in their polarity: 5′-3′ and 3′-5′, respectively. It is hard to envision how two proteins with opposing polarities may achieve the same outcome. Recently, two new activities were reported to be associated with WRN and BLM proteins, namely, strand-annealing and strand exchange activities on short, DNA oligonucleotide duplexes (25–27). The possibility that these activities might be involved in the rescue of *dna2-1* mutants by BLM and WRN led us to examine if Dna2 has similar activities. Indeed, we show herein, that both yeast and human Dna2 proteins are proficient in single strand annealing and ATP-independent strand exchange reactions. Based on the shared annealing/exchange properties of Dna2, BLM, and WRN, we propose a model that suggests how the newly discovered activities may contribute to the efficient processing of flap structures in lagging strand replication intermediates. This model may reconcile the apparent paradox that two helicases of opposing polarities may perform redundant tasks leading to indistinguishable products.

EXPERIMENTAL PROCEDURES

Recombinant Protein Preparation—Human Dna2 and hDna2D294A, nuclease defective, were tagged with FLAG at the C terminus and His_6 at the N terminus, and the tagged proteins were expressed and purified using the pFASTBacHT Bac to Bac system (Invitrogen, Carlsbad, CA), as described previously (2). Recombinant yDna2 K1080E tagged at the N terminus with one HA tag and at the C terminus with a His_6 tag was expressed in yeast and purified as described previously for the wild-type Dna2 expressed in baculovirus, namely using Ni^{2+} affinity and MonoQ chromatography (9). Wild-type yDna2 was prepared as described previously (9). Recombinant yDna2 E675A protein with His_6 tag at the C terminus was cloned in pFASTBacHT leaving only the C-terminal tag. This protein was purified as follows. 1 liter of Sf9 cells in TNM-FH medium containing 10% fetal calf serum in spinner flasks at 27 °C were infected with recombinant virus at an MOI of 5 and harvested after 50 h. The cell pellet was lysed in 50 ml of buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% IGEPAL, 10% glycerol, and EDTA-free protease inhibitor tablet (Roche Applied Science)) for 1 h at 4 °C. The lysate was cleared and then bound to Ni-NTA beads for 1 h at 4 °C and separated by centrifugation. After washing three times with 30 ml of buffer B (buffer A + 10 mM imidazole) the beads were placed in a column and washed with 10 ml of buffer B. The yDna2 was eluted with 5 ml of buffer C (buffer A + 300 mM imidazole) and dialyzed overnight against buffer D (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% glycerol) and loaded onto a MonoQ column equilibrated with the same buffer. Elution was carried out with a linear gradient from 0 to 100% buffer E (25 mM Tris-HCl, pH 7.4, 600 mM NaCl, 1 mM EDTA, 10% glycerol) over 10 ml. The yDna2 was eluted at ~270 mM NaCl. The yDna2-containing fractions were pooled in two pools and dialyzed against buffer F (25 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA, 25% glycerol) for 5 h (2 × 2.5 h), aliquoted, and frozen down. If necessary, enzymes were further diluted to appropriate concentrations prior to use in buffer containing 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 10% glycerol, 0.5 M NaCl, and 0.02% Nonidet P-40.

DNA Substrates—All oligonucleotides were synthesized by Integrated DNA technologies (Coralville, IA). Oligonucleotides used for strand annealing, strand exchange, and helicase assays were: D4 (5′-AGGTCTCGACTAACTCTAGTCGTTGTTCCACCCGTCCACCCGACGCCACCTCCTG-3′), T2 (5′-GCAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGTCG-3′) and T4 (5′-GCAGGAGGTGGCGTCGGGTGGACGGGTGGAACAACGACTAGAGTTAGTCGAGACCTATTGAAATTTAGGCTGGCACGGTCG-3′). 5′-Labeling of D4 was performed as described previously (5). For strand exchange assays, D4 and T2 were annealed at a molar ratio of 1:1 to make fork substrate. D4 and T4 were annealed in the same way to generate the marker that indicates the position of D4:T4 reaction products.

Strand Annealing Assay—Strand annealing activity of recombinant Dna2 was measured using a standard reaction mixture (20 μl) containing 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, the 5′-labeled D4 oligonucleotide (20 fmol), unlabeled T4 oligonucleotide (20 fmol), and various concentrations of $MgCl_2$ and ATP as indicated in figure legends. After incubation at 37 °C, reactions were stopped with 6× stop solution (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol, and 60 nM unlabeled D4 oligonucleotide). The reaction products ware separated with native PAGE (8–12%, 1× TBE) for 3 h at 4 °C, and analyzed using a Storm 860 PhosphorImager.

Strand annealing activity of yDna2 with 1.3-kb linear double-stranded DNA was measured using the same buffer conditions as above. The fragment used in these assays was obtained by PCR amplification using pBluescript ll SK (+) -hDna2 (21) as a template. 5′-ATGCTGCCCAAAATAGAAGAAG-3′ and 5′-CCCATCAACCAAGGATTATCAG-3′ primers were used in standard PCR reactions. 5′ labeling of the fragment was performed using T4 polynucleotide kinase (PNK) (5). Substrate dissolved in water was denatured by boiling (4 min) and quick

cooled on ice. 4 fmol of substrate was incubated with recombinant yDna2 (0–600 fmol, as indicated in the figure legends). After 5 min, the reaction was stopped by adding 6× stop solution (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol), and products were analyzed by 1% (w/v) agarose gel electrophoresis using 1× TAE buffer. Gels were dried and bands quantified as described above.

Strand Exchange Assays and DNA Helicase Assay—Strand exchange activity of recombinant Dna2 was measured using the same buffer conditions as strand annealing. For DNA substrate, radiolabeled D4:T2 fork substrate (5 fmol), and unlabeled T4 trap oligonucleotide (0–25 fmol, as indicated in figure legends) were used. Reactions were stopped by 6× stop solution, and reaction products were separated using 12% native PAGE. To see the DNA helicase activity of yDna2, T4 trap strand was omitted from the reaction (Fig. 2*C*).

For strand exchange assays on long DNA substrates, linear øX174 double-stranded DNA was treated with exonuclease III (Exolll) (28). øX174 RF1 DNA (New England Biolabs, Ipswich, MA) was cut with XhoI and then resected with Exolll (New England Biolabs, Ipswich, MA) in the buffer supplied by the manufacturer. Resection with Exolll was carried out at 37 °C at a ratio of 100 units Exolll per 3 μg of XhoI-cut linear double-stranded DNA. The extent of 3′-strand degradation was monitored on 1% agarose gels, in the presence of ethidium bromide, after 2, 6, 10, and 14-min incubation. After 2 min, the molecules were shortened by ~500–600 bases, and DNA from this time point was used as the duplex substrate in strand exchange assays. After resection, reaction products were deproteinized by phenol-chloroform treatment and ethanol-precipitated. 5′ Labeling of the fragment was performed using T4 PNK.

Strand exchange assays were performed using Exolll-treated linear øX174 double-stranded DNA and øX174 single-stranded circular DNA (New England Biolabs, Ipswich, MA) as described by others (Ref. 29 for example), with some modifications. Double-stranded DNA (0.1 fmol) was incubated with 0.5 fmol of single-stranded DNA in the strand exchange reaction buffer described above, with or without Mg^{2+} and ATP as indicated in figure legends. After 20 min, the reaction was stopped by addition of 6× stop solution (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol, 0.5μg/μl proteinase K). After 1 h at 55 °C, products were separated by 1% (w/v) agarose gel electrophoresis using 1× TAE buffer. Gels were dried and analyzed as above. To determine the mobility of expected intermediates, DNA strand exchange and annealing reactions were carried out in the absence of protein by incubating boiled or native Exolll-treated linear øX174 double-stranded DNA (0.1 fmol) with increasing amounts of øX174 single-stranded DNA (0–100 fmol). After overnight incubation at 65 °C, reaction products were analyzed by agarose gel electrophoresis.

DNA Binding Assay—The DNA binding activity of recombinant Dna2 was measured using a gel shift assay. The reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 8.0, 55 mM NaCl, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 10% glycerol, 1 mM EDTA, 5 fmol of radiolabeled DNA substrates, and various concentrations of $MgCl_2$ and ATP as indicated in figure legends. After incubation for 30 min at 4 °C, reaction mixtures were separated with native PAGE (12%, 1× TBE) for 3 h at 4 °C, and analyzed using a Storm 860 PhosphorImager.

RESULTS

Single Strand Annealing Properties of Yeast and Human DNA2—Strand annealing reactions were performed using two complementary oligonucleotides, designated D4 and T4 (see "Experimental Procedures"), as shown in Fig. 1*A*. Except where otherwise noted, the nuclease-defective forms of yDna2 and hDna2 were used to minimize destruction of the substrates used. As shown in Fig. 1*B*, both yDna2 and hDna2 strongly stimulate the annealing of the two oligonucleotides. Notably, in the case of the yeast enzyme, the strand annealing is saturated at equimolar levels of the protein and DNA (see *lanes 5* and *6*).

The yeast protein is efficient in strand annealing both in the absence and in the presence of 4 mM Mg^{2+} (Fig. 1*B*, *lanes 2–11*). Promotion of annealing by the human protein, in contrast, is dependent on the presence of Mg^{2+} (Fig. 1*A*, *lanes 12–21*). Thus, both proteins are efficient in strand annealing, though with different response to divalent cation. In the case of yDna2, in the absence of Mg^{2+}, there seems to be a threshold concentration (Fig. 1*B*, *lanes 4* and *5*) at which strand annealing occurs, rather than a gradual increase with increasing enzyme concentration, as is seen with the hDna2. We also examined the effect of NaCl on strand annealing by yDna2. As shown in Fig. 1*C*, NaCl concentration above 100 mM was inhibitory.

Next, we examined the effect of ATP on the strand annealing activity of yDna2. As shown in Fig. 1*D*, ATP is inhibitory to strand annealing at all concentrations tested, and inhibition is proportional to ATP concentration. hDNA2-promoted strand annealing is also inhibited in the presence of ATP (Fig. 1*E*). ADP does not inhibit the strand annealing activity of yDna2 (data not shown).

We also examined the effect of a point mutation in the yDna2 helicase domain (K1080E, Walker A box) on strand annealing. This mutant protein has no ATPase or helicase activity but retains normal levels of nuclease activity (9). Notably, the yeast helicase mutant is efficient in promoting strand annealing, suggesting that ATP hydrolysis is not important for this activity of yDna2 (and also verifying that strand annealing can occur in the presence of an intact nuclease domain) (Fig. 1*D*, *lanes 17–26*). Dispensability of ATPase is not surprising, since, as shown above, strand annealing by yDna2 does not require Mg^{2+}, which would be expected to be an essential cofactor in ATP binding and hydrolysis (Fig. 1*B*). Unexpectedly, however, ATP (1 mM) inhibits strand annealing even in the K1080E mutant. Thus, the Walker A motif, K1080E mutant protein, though defective in ATP hydrolysis (Fig. 1 and Ref. 9), can apparently interact with ATP. In summary, strand annealing is Mg^{2+}-independent for yDna2 but Mg^{2+}-dependent for hDna2. The yeast protein is inhibited by NaCl, and both yeast and human proteins, even in the Walker A box mutant, are inhibited by ATP. Strand annealing does not require either nuclease or helicase activities of Dna2.

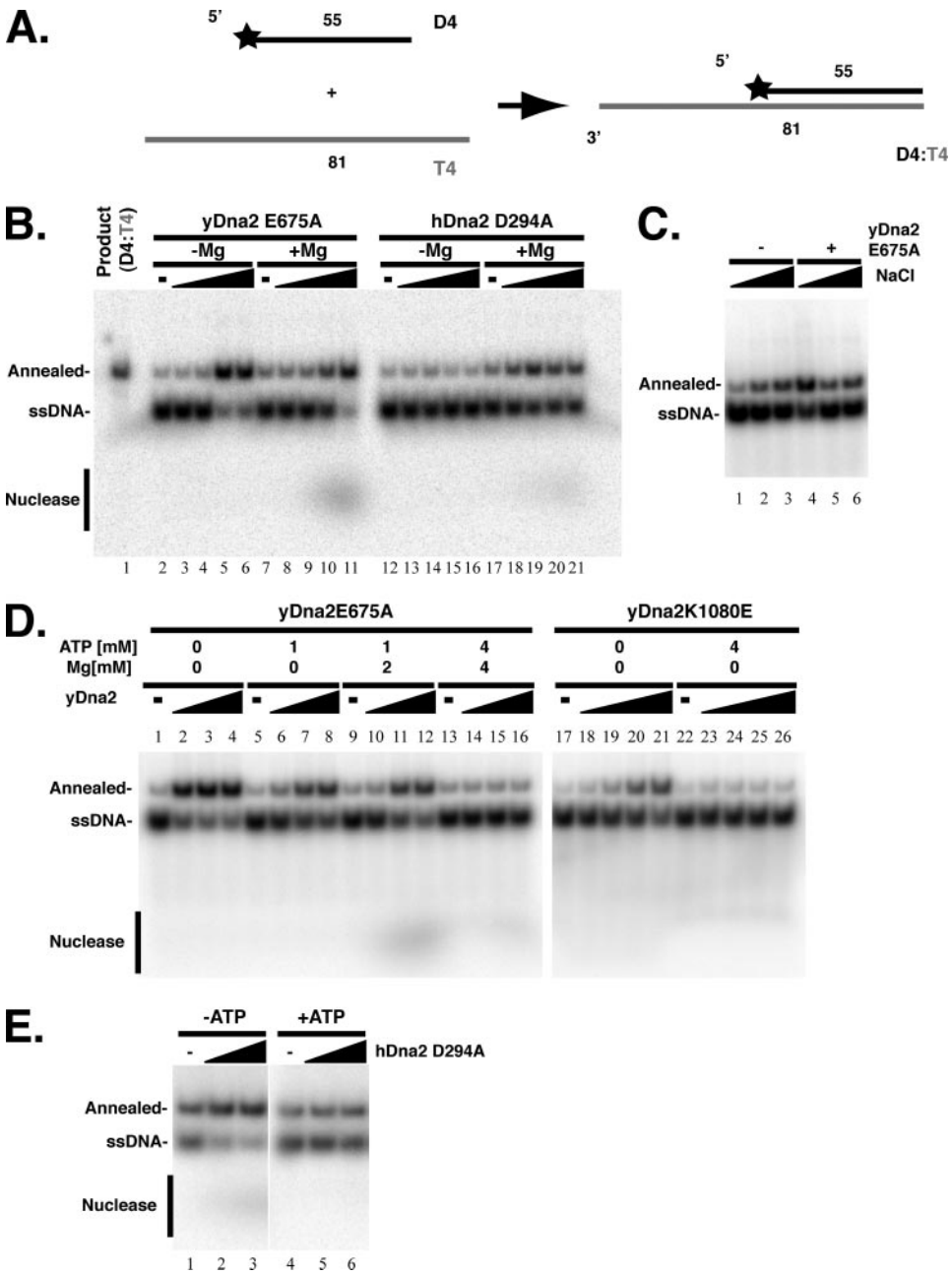

FIGURE 1. **Strand annealing activity of human and yeast Dna2.** *A*, strand annealing activity of recombinant Dna2 was measured as annealing of two ssDNA molecules to generate a partially single-stranded DNA duplex (*D4:T4*) as shown in the schematic diagram. *Asterisks* indicate the positions of radiolabels. *B*, effect of Mg^{2+} on strand annealing activity of Dna2. Increasing amounts (0, 25, 100, 300, 600 fmol for hDna2 D294A and 0, 1, 5, 25, 100 fmol for yDna2 E675A, as indicated by *triangles*) of recombinant, nuclease-deficient Dna2 were incubated with 20 fmol of ssDNA substrates at 37 °C as described under "Experimental Procedures." After 10 min, reactions were stopped by adding stop solution, and reaction products were separated by native PAGE. In *lanes 7–11* and *17–21*, reaction mixtures contained 4 mM Mg^{2+}. *Lane 1* provides the marker for the annealed product, as indicated. Positions of ssDNA D4 substrate (ssDNA), annealed products (*Annealed*) and nuclease products (*Nuclease*) are indicated on the left. *C*, effect of NaCl on the strand annealing activity of Dna2. Reactions contained yDna2 E675A (25 fmol) and 5 fmol substrate in the presence of increasing concentrations of NaCl (25, 100, and 200 mM, as indicated by *triangles*). Mg^{2+} was omitted from the reaction. *D*, effects of ATP and Mg^{2+} concentration on the strand annealing activity of yDna2 mutants. Strand annealing activity of increasing amounts (0, 25, 100, and 170 fmol for yDna2 E675A, and 0, 5, 25, 100, and 350 fmol for yDna2 K1080E) were investigated using ssDNA substrates (5 fmol) in the presence of various concentrations of ATP and Mg^{2+} as shown in the figure. After 10 min of incubation, reaction products were investigated as in *B*. *E*, effect of ATP on strand annealing activity of hDna2. Increasing amounts (0, 25, 100 fmol) of nuclease negative hDna2 D294A were incubated with 20 fmol of ssDNA substrates at 37 °C for 30 min, reactions were stopped by adding stop solution and reaction products were separated by native PAGE. In *lanes 1–3*, reaction mixtures contained 4 mM Mg^{2+}. In *lanes 4–6*, reaction mixtures contained 4 mM Mg^{2+} and 4 mM ATP.

Strand Exchange Promoted by Yeast and Human DNA2—In the following experiments, we tested if yDna2 and hDna2 are proficient in strand exchange. Oligonucleotides D4 and T2 (see list of oligonucleotides under "Experimental Procedures") were annealed at a 1:1 ratio. The oligonucleotides form a fork molecule with 5′ and 3′ single-stranded tails adjacent to the duplex region (Fig. 2*A*). The 5′-tail is the preferred substrate for yDna2 (8, 30). Reactions were performed in the presence or absence of a third oligonucleotide (T4), with sequences complementary to D4. If strand exchange occurs, it will result in the D4:T4 product. As shown in Fig. 2*B*, yDna2 protein catalyzes strand exchange, and, as with the strand annealing activity, the strand exchange occurs both in the presence and the absence of Mg^{2+} ion. We do observe a slight reduction in strand exchange product in the presence of Mg^{2+}, but we attribute the reduction in exchanged product (*lanes 12–14* compared with *lanes 6–8*) to the residual nuclease activity of the protein in the presence of Mg^{2+}. We suggest that the Dna2 nuclease acts on the long 5′-tail of the fork, thus reducing the amount of substrate. Furthermore, we suggest that the product of strand exchange is stable and readily detected because the 3′-tailed oligonucleotide is more resistant to the Dna2 nucleolytic cleavage.

Additionally, we examined the effect of adding Mg^{2+}/ATP into the reactions. Nuclease activity is known to be inhibited under these conditions (30, 31). As seen in Fig. 2*B*, *lanes 18–20*, these conditions promote Dna2 helicase and unwinding of the forked substrate. Clearly, there is an increase in the unwound product between 5 and 15 min (Fig. 2*B*, compare *lanes 18* and *19*) followed by a decrease in the unwound product (*lane 20*). Also, the helicase assay in the absence of T4 clearly shows a gradual increase of the unwound product during the course of the experiment (Fig. 2*C*). In contrast to unwound product,

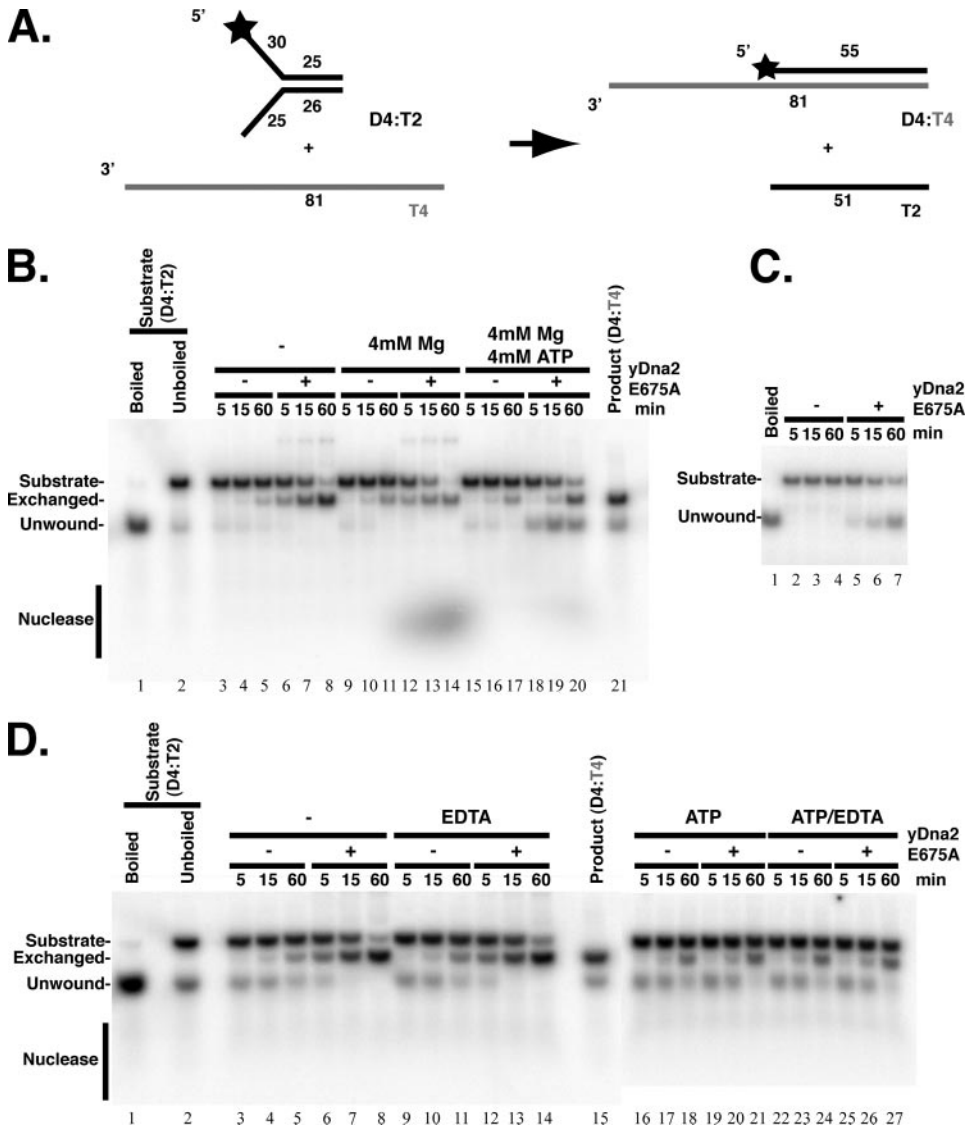

FIGURE 2. **Strand exchange activity of yDna2.** *A*, schematic diagram of the strand exchange reactions between a labeled fork substrate (*D4:T2*) and a complementary 81-mer (T4). The products are labeled, partially duplex DNA and unlabeled 51-nt oligomer. *B*, time course of strand exchange by yDna2 E675A. A labeled fork substrate (D4:T2, 5 fmol) was incubated with yDna2 E675A (100 fmol) or without yDna2 in the presence of 25 fmol complementary strand (T4). Reactions were stopped at indicated times by adding stop solution, and reaction products were analyzed as described under "Experimental Procedures." Reaction mixtures contained no Mg^{2+} or ATP (*lanes 3–8*), 4 mM Mg^{2+} (*lanes 9–14*), or 4 mM Mg^{2+} and 4 mM ATP (*lanes 15–20*). Substrate (*Unboiled*, *lane 2*), boiled substrate (*Boiled*, *lane 1*), and D4:T4 (*Product*, *lane 21*) were also loaded to show the position of substrate, unwound substrate, and reaction product, respectively. *C*, DNA helicase activity of yDna2. Fork substrate (D4:T2, 5 fmol) was incubated with or without yDna2 at 37 °C for indicated times, and unwinding of fork substrate was detected by native PAGE. *D*, effect of ATP and EDTA on the strand exchange activity of yDna2. Time course of strand exchange was investigated using 100 fmol of yDna2 E675A as described in *B*. Reaction mixtures contained 4 mM EDTA (*lanes 9–14*), 4 mM ATP (*lanes 16–21*), or 4 mM ATP and 4 mM EDTA (*lanes 22–27*).

there is no detectable strand exchange product at the 5 min time point in the presence of ATP (Fig. 2*B*, *lane 18*). We propose that strand exchange is inhibited under conditions favoring the helicase activity, that is, in the presence of 4 mM ATP and 4 mM Mg^{2+} and that the band at the position of apparent strand exchange (*lane 20*) is due to a two step process consisting of an unwinding event followed by spontaneous, protein-independent strand annealing of the unwound D4 and the T4. Consistent with this interpretation, we find that ATP inhibits the strand exchange reaction (Fig. 2*D*). As also shown above (Fig. 2*B*), strand exchange is efficient in the absence of Mg^{2+} (Fig. 2*D*, *lanes 6–8* and *12–14*). ATP, however, even in the absence of Mg^{2+}, and thus in the absence of helicase activity, is inhibitory to strand exchange (Fig. 2*D*, *lanes 19–21* and *25–27*). EDTA was added to the reactions to chelate any contaminating metals.

hDna2 is remarkably similar to yDna2 in the strand exchange reactions. As shown in Fig. 3, *A* and *B*, like yDna2, hDNA2 promotes strand exchange in the absence, as well as in the presence of Mg^{2+}. Because the strand exchange activity observed is weaker than that in the yeast protein, however, we tried to optimize the conditions for strand exchange by increasing the concentration of the T4 acceptor oligonucleotide. As seen in Fig. 3*B*, 5-fold molar excess of T4 oligonucleotide clearly enhances the strand exchange reaction. We have reproduced these results in a time course experiment under optimal T4 concentrations (Fig. 3*C*). Both in the presence and the absence of Mg^{2+}, nearly all of the substrate was converted to the strand exchange product within 60 min (Fig. 3*C*, *lanes 9* and *15*). Again, as with yDna2, in the presence of Mg^{2+}, residual nuclease activity reduces the amount of strand exchange product, probably reflecting degradation of the labeled substrate (Fig. 3*C*, compare *lanes 7–9* and *13–15*). Addition of Mg^{2+}/ATP to the reaction at concentrations promoting helicase activity of hDNA2 appears to inhibit strand exchange, significantly reducing the D4:T4 product observed (Fig. 3*C*, compare *lanes 7–9* with *lanes 19–21*). Because of the weak unwinding activity of hDNA2 (Fig. 3*C*, *lanes 19* and *20*) compared with yDna2 helicase, the inhibitory effect of ATP is unlikely attributed to competing unwinding reactions and more likely to an inhibitory effect of ATP on the strand exchange itself. The inhibitory effect of ATP and independence of Mg^{2+} argues that the strand exchange being catalyzed is a one step reaction rather than an energy-dependent unwinding event followed by annealing.

Effect of ATP on Interaction of Dna2 with DNA—ATP inhibits the nuclease, strand annealing, and strand exchange activities of Dna2. This suggests that ATP binding alters the interac-

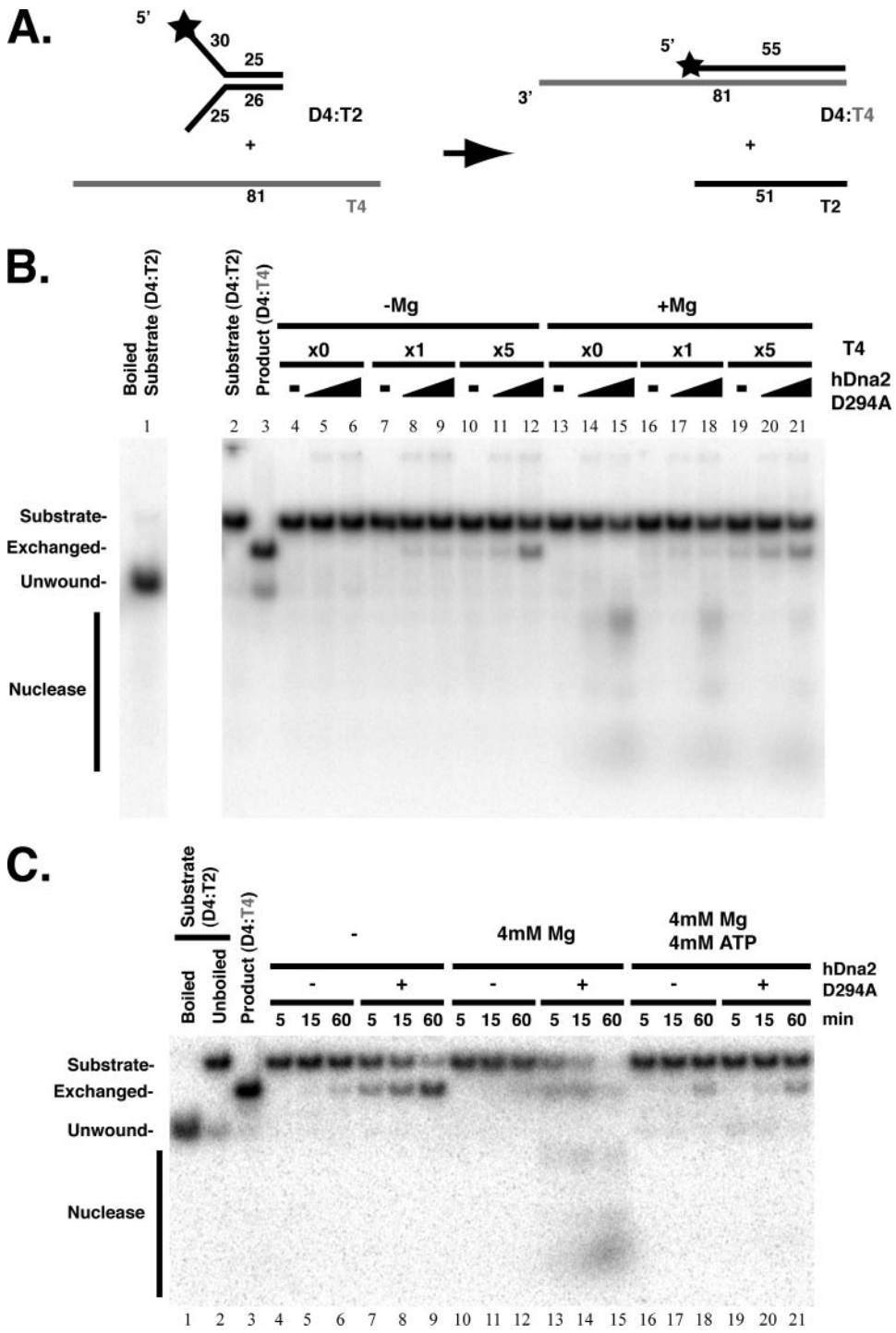

FIGURE 3. **Strand exchange activity of human Dna2.** *A*, schematic diagram of the strand exchange reactions between a labeled fork substrate (*D4:T2*) and a complementary 81-mer (T4). The products are labeled partially duplex DNA and unlabeled 51-nt oligomer. *B*, effect of the concentration of trap strand on strand exchange activity of hDna2. Strand exchange activity of increasing amounts (0, 400, 1300 fmol, as indicates by *triangles*) of human Dna2 D294A was investigated using labeled fork substrate (D4:T2, 5 fmol) in the presence of 0 fmol (×0), 5 fmol (×1) and 25 fmol (×5) of complementary T4 as described in the legend to Fig. 2. In *lanes 13–21*, reaction mixtures contained 4 mM $^{2+}$. *C*, time course of strand exchange. A labeled fork substrate (D4:T2, 5 fmol) was incubated without or with Dna2 D294A (1300 fmol) in the presence of 25 fmol complementary strand (T4). Reactions were stopped at indicated times and reaction products were analyzed by native PAGE. Reaction mixtures contained 4 mM Mg^{2+} (*lanes 10–15*) or 4 mM Mg^{2+} and 4 mM ATP (*lanes 16–21*).

tion of Dna2 with the DNA substrate. To test this idea, we monitored the binding of yDna2E675A to single-stranded DNA, to a fork molecule, and to a partially single-stranded duplex molecule (Fig. 4*A*). ATP (4 mM) clearly inhibits the binding of Dna2 to each of these substrates (Fig. 4*B*), both in the presence and in the absence of Mg^{2+}. The negative effect on the interaction of Dna2 with DNA may account for the inhibitory effects of ATP on the multiple activities of Dna2.

Strand Annealing and Strand Exchange Analysis with Long DNA Substrates—To determine if Dna2 can promote strand annealing and strand exchange on longer substrates, we used a 1.3-kb duplex DNA fragment generated by PCR for strand annealing and a øX174 based system for strand exchange experiments. Yeast Dna2 did not promote annealing of the separated strands of the 1.3-kb fragment under the conditions tested, either when present at low (catalytic) or high (stoichiometric) concentrations (Fig. 5*A*).

For the strand exchange assay, we first created linear duplex øX174 DNA with single-stranded 5′ termini to allow Dna2 binding. The expected products and intermediates of strand exchange between this linear duplex and single-stranded circular øX174 DNA are shown in Fig. 5*B*. Strand exchange products (S.E. in the figure) would migrate as sigma, alpha, or gapped circular forms depending on the extent to which exchange could occur. Incubation results in annealing of the 5′ single-stranded tails to the circular single-stranded molecules and formation of an intermediate products visualized as slowest migrating species in the controls shown in Fig. 5*C*, *lanes 4* and *5*. Boiling and slow cooling of a mixture of the native, tailed duplex øX174 with the circular øX174 results in the formation of labeled, gapped double-stranded molecules with faster electrophoretic mobility than the intermediate products but slower than the single-stranded circular DNA (Fig. 5*C*, *lanes 8* and *9*), as determined by ethidium bromide staining of control gapped DNA circles (not shown). Complete strand exchange gives rise to labeled gapped circular molecule is the expected strand exchange product. Fig. 5*D* shows that

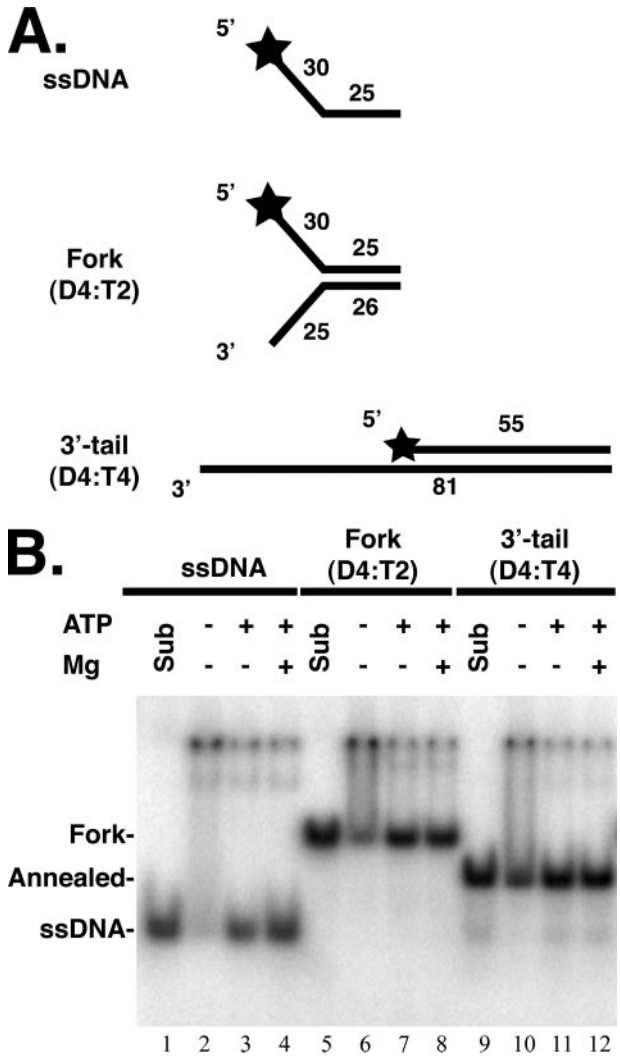

FIGURE 4. **Effect of ATP on the DNA binding activity of yDna2.** *A*, DNA substrates used for DNA binding assay. *B*, various radiolabeled DNA substrates shown in *A* (each 5 fmol, *lanes 1–5*, single-stranded DNA; *lanes 5–8*, fork structure; *lanes 9–12*, 3′-ssDNA tailed duplex) were incubated with or without yDna2 E675A for 30 min on ice. The protein-DNA complexes were then resolved by native PAGE, and radiolabeled bands were detected with PhosphorImager. 4 mM ATP and/or Mg^{2+} were included in the reaction mixture as indicated.

Dna2 fails to promote strand exchange either in the absence or presence of ATP at either catalytic, stoichiometric (with respect to total single-stranded DNA), or intermediate yDna2 protein concentrations. Also, it does not promote annealing of the 5′ protruding tails to produce intermediate forms. We conclude from these studies that Dna2, at least under standard strand exchange conditions, does not promote strand exchange over extensive lengths of DNA.

DISCUSSION

In this report we describe two new and conserved activities of Dna2: strand annealing and ATP-independent strand exchange on short, but not on long, DNA duplexes. Enzymes from both yeast and human cells appear to have similar efficiency. yDna2 does not require Mg^{2+} for strand annealing, although the strand annealing reaction of hDna2 is Mg^{2+}-dependent. Rates of strand exchange by either enzyme, however, are not affected by Mg^{2+}. ATP inhibits both strand annealing and strand exchange for both enzymes (under all ionic conditions). This is interesting in view of the fact that ATP has been shown previously to inhibit the nuclease activity of Dna2, as well. DNA binding studies suggest that ATP alters the interaction of yDna2 with single-stranded DNA. Taken together the inhibitory effect of ATP on both activities, albeit at high concentrations, suggests that ATP binding may lead to a conformational switch in the enzyme that favors one or the other of its various activities. Finally, continued inhibition of annealing by ATP in the K1080E mutant, which should be deficient in binding the γ-phosphate of ATP, suggests that Dna2 may contain an additional ATP binding site. However, we have not been able to date by simple motif searches to identify such a site, so the mutation may not abolish ATP interaction.

The oligonucleotide assays used here differ from those used to characterize both the classical ATP-dependent and more recently discovered ATP-independent strand exchange activities involved in various cellular and viral recombination pathways (32). Some, in particular those observed with prokaryotic proteins, but not all of these pathways involve formation of potential helical protein/DNA filaments as intermediates in the reactions (32). The oligonucleotides used in our assays are too short to allow such structures to form. Therefore, we attempted to show strand annealing and strand exchange on longer substrates. These attempts were unsuccessful, however (Fig. 5). This does not exclude the possibility that under different experimental conditions, possibly involving additional proteins and/or nucleotide cofactors other than ATP, Dna2-mediated strand annealing and strand exchange activities could be detected on longer substrates. On the other hand, it is possible that Dna2 specifically evolved to act on shorter substrates, which may commonly occur during Okazaki fragment processing.

To fully understand the mechanism by which Dna2 contributes to flap processing during DNA replication and repair, all of the biochemical activities of this multifunctional protein must be taken into account, including the newly revealed strand annealing and strand exchange. It is believed that the essential function of *DNA2* in lagging strand DNA synthesis is to cleave long flaps, which may arise due to polymerase errors, perhaps facilitated by impaired FEN1 function. FEN1, the major nuclease in Okazaki fragment processing, cleaves long flap substrates with low efficiency in the presence of RPA. Overexpression of FEN1 *in vivo* or higher concentration of the protein in the *in vitro* reactions alleviates this problem, as does addition of Dna2, which in contrast to FEN1 is stimulated by RPA (5). It has thus been proposed that the nuclease activity of Dna2 provides a substrate utilized by FEN1. The Dna2 helicase may be required for resolution of secondary structures that may arise in long flaps, particularly in regions of repetitive sequence, and the nuclease might also be required to remove the sequences that can form secondary structure to allow for FEN1 cleavage (5). While it has been easy to show that Dna2 stimulates FEN1 in reconstituted reactions, experiments trying to establish the coordination of FEN1/Dna2/RPA interactions are so far inconclusive, raising the issue of how stimulation occurs. Evidence that Dna2 and FEN1 interact physically supports coordination

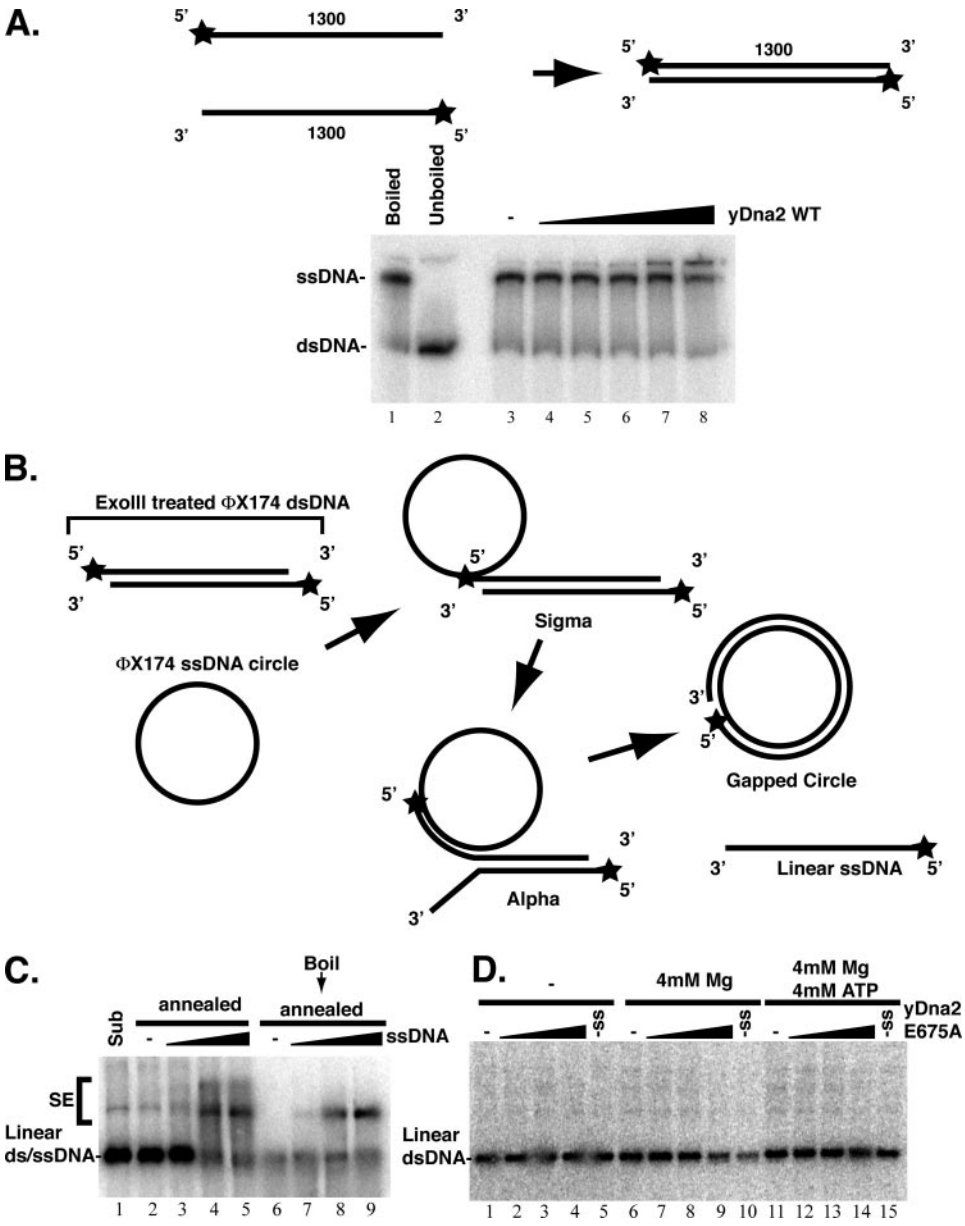

FIGURE 5. **Strand annealing and strand exchange assays with long DNA substrates.** *A*, strand annealing assay with yeast Dna2 using 1.3-kb DNA substrate. Strand annealing activity of recombinant wild-type yDna2 was measured as annealing of ssDNA molecules of 1.3 kb, prepared as described under "Experimental Procedures," as shown in the schematic diagram. Increasing amounts of recombinant yDna2 (0, 5, 25, 100, 300, 600 fmol, as indicated) were incubated with 4 fmol of ssDNA substrates in the absence of Mg^{2+}/ATP. After 5 min at 37 °C, reactions were stopped by adding stop solution and reaction products were separated by 5% native PAGE. *Lane 1* is substrate only control. *Lane 2* is native substrate to show the position of annealed product. Positions of ssDNA substrate (*ssDNA*), and annealed product (*dsDNA*) are indicated on the *left*. *B*, diagram of the expected strand exchange products in reactions containing ExoIII-treated øX174 double-stranded DNA and øX174 single-stranded circular DNA. Sigma, alpha, and gapped circles represent strand exchange intermediates and products expected at various stages of the reaction. *C*, spontaneous strand exchange reaction in the absence of Dna2. ExoIII-treated øX174 double stranded DNA (0.1 fmol) was mixed with increasing amounts of single-stranded circular øX174 DNA (0, 0.5, 10, and 100 fmol, *lanes 2–5* and *6–9*). Native (*lanes 2–5*) or boiled (*lanes 6–9*) samples were then incubated at 65 °C overnight, and reaction products were analyzed as described under "Experimental Procedures." Positions of linear DNA and strand exchange products (*SE*) are shown on the *left*. *Lane 1* is native substrate without 65 °C incubation. Markers were linear ds øX174 DNA, ss øX174 DNA, and gapped circular øX174 stained with EtBr but not shown. *D*, strand exchange assay with yeast Dna2 using long DNA substrates. The labeled ExoIII-treated øX174 double-stranded (0.1 fmol) and øX174 single-stranded circular DNA (0.5 fmol) were incubated with increasing amounts of yDna2 E675A (0, 1.7, 17, 170 fmol, *lanes 1–4*, *6–9*, and *11–14*) at 37 °C for 20 min, and reaction products were analyzed as described under "Experimental Procedures." *Lanes 1–5*, buffer only, *lanes 6–10*, reaction mixtures contained 4 mM Mg^{2+}, *lanes 11–15*, reaction mixtures contained 4 mM Mg^{2+} and 4 mM ATP. As control, yDna2 E675A (170 fmol) was incubated with the labeled substrate in the absence of single-stranded circular DNA (*lanes 5*, *10*, and *15*).

in some fashion. Importantly, it is not known whether cleavage of the long flaps by Dna2 leads to a substrate captured and cleaved by FEN1 or Dna2 stimulates cleavage by FEN1 *per se*. Under one set of conditions, Dna2 products do appear before FEN1 products, but in other studies only at high concentrations of yDna2 do the short flaps resulting from Dna2 cleavage appear to serve as favorable substrates for FEN1 (5). Thus, the nuclease and helicase activities of Dna2 may not fully account for stimulation of FEN1.

Recent evidence has underscored the fact that the preferred substrate for FEN1 *in vivo* is not a single-stranded 5′ flap, but rather a double flap structure with equilibrating 3′ and 5′ flaps (see Fig. 6). FEN1 favors cutting a substrate having a short 5′-flap, <30 nt, and 1 nt 3′-flap and cleaves 1 nt downstream of the base of the 5′-flap. Reannealing of the 3′-flap then allows ligation. Equilibrating flaps are proposed to arise by generation of a 5′-flap by polymerase δ strand displacement activity followed by a reversible 3′ fraying and 3′- or 5′-flap realignment. While this may not occur at every Okazaki fragment, it is likely to arise if there is a pol δ/FEN1 imbalance and cleavage is not immediate (12, 33, 34). yDna2, at high protein concentrations, stimulates FEN1 cleavage of such flaps (5), and hDna2 was shown to stimulate FEN1-mediated formation of a ligatable nick in equilibrating flap structures (3). Interestingly, RPA had no effect on the outcome of the latter reaction (3). Moreover, the nucleolytic products of hDna2 do not seem to contribute significantly to the FEN1 reactions products, though the intermediates were not analyzed in sufficient detail to eliminate this possibility entirely. yFEN1 is stimulated by Dna2 only at high Dna2 protein concentrations in our hands (5). This may be attributed to the fact that this stimulation has always been measured in the presence of high levels of ATP and that the Dna2-promoted strand annealing

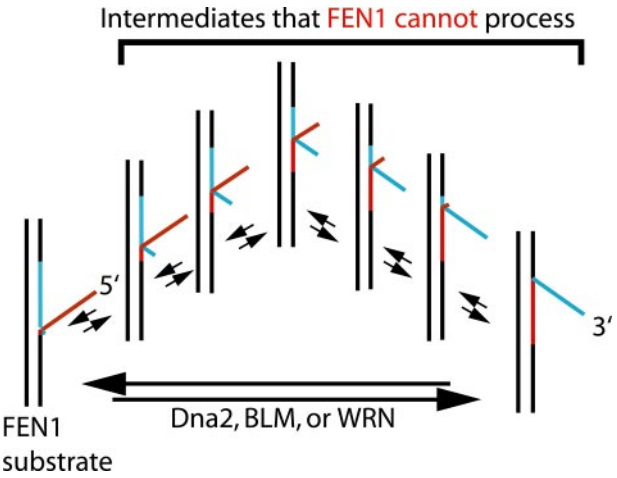

FIGURE 6. **Equilibrating flap intermediates in Okazaki fragment synthesis and RNA/DNA primer removal.** Shown on the *left* is the optimal substrate for FEN1. Dna2, BLM, or WRN could increase the frequency of appearance of the extreme right and left substrates through their strand annealing, strand exchange activities. Structures with 3′-flaps could be processed by the 3′-exonuclease of pol δ, accounting for the lethality of *rad27 pol3-01* and *dna2 pol3-01*. See text for further details.

activity is strongly inhibited in the presence of ATP. Only at high Dna2 concentrations can Dna2 promote strand annealing in the presence of ATP (Fig. 1*D*). Dna2/FEN1 interactions have mostly been analyzed in conditions favoring both the helicase and nuclease activities of Dna2, ATP/Mg^{2+}, 2:1 and ATP/Mg^{2+}, 1:2, respectively. Because absence of ATP seems to favor the strand annealing activity, it will be interesting to reexamine the Dna2/FEN1 experiments with no ATP present in the reactions. Stimulation of FEN1 cleavage under these conditions would provide an explanation for the result that Dna2 was stimulatory only at high protein concentration in the presence of ATP. It is interesting to note that ATP was found to be inhibitory to binding of Dna2 to single-stranded DNA. This may suggest that ATP induces a conformational change in the protein, possibly changing the specificity of the protein. Future experiments will certainly lead to refinements of this heuristic model.

The strand-annealing hypothesis may also enlighten our understanding of the relationship between Dna2 and the WRN and BLM helicases. Recent reports have shown that WRN helicase/nuclease and BLM helicase, like Dna2, also have single-strand annealing and strand exchange activity. Both BLM and WRN, also like Dna2, are known to interact with FEN1 and to stimulate FEN1 cleavage *in vitro*. The mechanism of FEN1 stimulation remains elusive in all these cases. It was proposed that BLM- and WRN- mediated stimulation of FEN1 may be due to local increased concentration of FEN1 at the fork. BLM and WRN were also shown to suppress the temperature sensitive growth of a *dna2* mutant strain, suggesting that BLM, WRN, and Dna2 have either compensating or redundant functions. Table 1 lists the activities of Dna2 genes from different species, BLM and WRN. The BLM and WRN genes suppress the Dna2 mutation despite deficiency in either helicase or nuclease. Strand annealing and strand exchange activities are common to all gene products. It is intriguing that the expression of the C-terminal domain of WRN, with no helicase or

TABLE 1

Genes suppressing Dna2 defects and their biochemical characteristics

Suppression may not require helicase activity (as in $WRN_{940-1432}$) or nuclease (BLM and $WRN_{940-1432}$). A common feature of Dna2, BLM, and WRN are strand annealing/strand exchange activities, and in the case of $WRN_{940-1432}$, these are sufficient for suppression of a Dna2 defect.

Gene	Suppression of *dna2* defect	Helicase	Nuclease	Annealing/ exchange
yDna2	+[a]	+	+	+
hDna2	+	+	+	+
XDna2	+	ND[b]	+	NT[c]
BLM	+	+	−[d]	+
WRN	+	+	+	+
$WRN_{940-1432}$	+	−	−	+

[a] +, yes.
[b] ND, not detected.
[c] NT, not tested.
[d] −, no.

nuclease function, is sufficient to suppress *dna2*, suggesting the helicase activity is not required for suppression. The C-terminal WRN domain is responsible for the FEN1 interaction as well as strand annealing/strand exchange functions (22). In BLM, it was reported that the helicase domain may be important for *dna2* suppression, since overexpression of a mutant protein that lacked helicase did not suppress the growth defect of a *dna2-1* mutant strain at 37 °C (21). However, it is noteworthy that partial suppression was observed at 35 °C, well above the 30 °C maximum permissive temperature of the *dna2-1* mutant by itself. It is possible that lack of full suppression of *dna2* (at 37 °C) by BLM K695T was caused by a defect in another of the activities of BLM, perhaps arising due to lower expression of the BLM K695T protein, lower stability of the protein, or misfolding, rather than to the helicase defect *per se*.

How can the type of strand annealing/strand exchange activities described here contribute to Okazaki fragment processing? The role of Dna2 has often been ascribed to removal of secondary structures during processing of repetitive DNAs. The new activities may suggest the opposite; that they can catalyze formation of structures that lead to repair. More likely, as suggested in Fig. 6, we propose that the annealing/strand exchange activities of Dna2, BLM, and WRN may affect the kinetics of flap equilibration (the 3′ and 5′ fraying and/or 3′ and 5′ realignment steps). As a result, more intermediates with the appropriate configuration for FEN1 and/or pol δ cleavage would arise at any given time leading to increased cleavage. Flaps with 3′-ends could be cleaved by either Dna2 or the 3′ exonuclease of pol δ. Thus, the stimulation by Dna2/BLM/WRN of FEN1 flap cleavage may, in addition to or in place of a protein/protein interactions and sequential nuclease activities (in the case of Dna2), simply reflect the increased availability of a favorable substrate.

Finally, strand annealing and strand exchange activities are most often associated with processing of intermediates in recombination rather than Okazaki fragment processing. The discovery of the activities described here may extend models for *in vivo* roles of Dna2 to formation and/or resolution of DNA structures that form upon failure of Okazaki fragment processing, stalling of the replication fork in the presence of template damage, or replication restart. Alternatively, they may be important for the telomeric role of Dna2 (4, 35).

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