

Dna2p Helicase/Nuclease Is a Tracking Protein, Like FEN1, for Flap Cleavage during Okazaki Fragment Maturation*

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Hui-I Kao^{‡§}, Judith L. Campbell[¶], and Robert A. Bambara^{‡¶}

From the [‡]Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and the [¶]Braun Laboratories, California Institute of Technology, Pasadena, California 91125

During cellular DNA replication the lagging strand is generated as discontinuous segments called Okazaki fragments. Each contains an initiator RNA primer that is removed prior to joining of the strands. Primer removal in eukaryotes requires displacement of the primer into a flap that is cleaved off by flap endonuclease 1 (FEN1). FEN1 employs a unique tracking mechanism that requires the recognition of the free 5' terminus and then movement to the base of the flap for cleavage. Abnormally long flaps are coated by replication protein A (RPA), inhibiting FEN1 cleavage. A second nuclease, Dna2p, is needed to cleave an RPA-coated flap producing a short RPA-free flap, favored by FEN1. Here we show that Dna2p is also a tracking protein. Annealed primers or conjugated biotin-streptavidin complex block Dna2p entry and movement. Single-stranded binding protein-coated flaps inhibit Dna2p cleavage. Like FEN1, Dna2p can track over substrates with a non-Watson Crick base, such as a biotin, or a missing base within a chain. Unlike FEN1, Dna2p shows evidence of a “threading-like” mechanism that does not support tracking over a branched substrate. We propose that the two nucleases both track, Dna2p first and then FEN1, to remove initiator RNA via long flap intermediates.

During cellular DNA replication, the lagging strand is primed frequently to generate many RNA-initiated DNA segments called Okazaki fragments. In eukaryotes, these segments are 100–150 nucleotides in length (1–3). The 8–12 ribonucleotide primers (4) must be removed prior to joining of the DNA segments to make the continuous lagging strand. The primer is cleaved off via a single-stranded flap intermediate, a structure that increases the accessibility of the primers to nucleases. The flap is created by the strand displacement synthesis activity of DNA polymerase δ and proliferating cell nuclear antigen (5–7).

Two nucleases that have been proposed to access and cleave flaps (6, 8–10). One is flap endonuclease 1 (FEN1).¹ FEN1 is a

structure-specific endonuclease that recognizes one intermediate of strand displacement. This is a double flap with a 1-nucleotide 3'-tail (11–13).

Biochemical analyses showed that FEN1 employs a unique tracking mechanism in which it recognizes the free 5'-end of the flap and then moves to the base of the flap for cleavage (14). Crystal structures of FEN1 and its homologues reveal a conserved flexible region, called the helical arch or loop domain, through which the flap was proposed to be threaded during tracking (15–18). The term “threading” is taken to mean that the flap passes through a fully enclosed hole in the protein. However, studies of substrate specificity indicated that the enzyme is able to cleave on a branched substrate that could not fit through the proposed hole (19). This indicates that FEN1 tracking does not employ a threading mechanism. The obligatory nature of tracking suggests that it is important for the protection of replication intermediates.

Studies of enzyme properties and reconstituted assays indicate that FEN1 prefers short flap substrates and that coordinated strand displacement and cleavage result in frequent cuts (6, 10). These results suggest that within the cell, flaps are removed when they are only a few nucleotides long. However, in some regions, stable structures can form within the flap as soon as the flaps arise, inhibiting the accessibility of the substrates to FEN1. These structures will inhibit FEN1 cleavage and allow the flap to get longer than normal (10, 20). In this situation, flaps may get long enough for RPA coating, which inhibits FEN1 tracking for cleavage. These flaps are proposed to require another protein for efficient metabolism, the Dna2 helicase/nuclease (10, 21).

Dna2p is a multifunctional enzyme that possesses 5'–3' helicase, DNA-specific ATPase, and single strand DNA-specific endonuclease activities (22). Dna2p is an essential gene in yeast, encoding a 172-kDa protein (9, 23, 24). Genetic analyses indicated that FEN1 and Dna2p interact with each other both physically and genetically, suggesting the two enzymes work at a similar step of DNA replication (9). In addition, Dna2p interacts with RPA biochemically and genetically, suggesting a model in which Dna2p and RPA process long flaps to produce short flaps that support cleavage by FEN1 (6, 10, 21). Because both Dna2p and FEN1 act on flap intermediates during replication, and a preference of both helicase and endonuclease activities of Dna2p for 5'-ends was reported (22, 25), we questioned whether Dna2p has evolved to track on flaps like FEN1.

Preliminary biochemical analyses indicated that Dna2p endonuclease activity prefers ssDNA as the point of entry, but the enzymatic activity is higher when a 5'-end rather than a 3'-end is available (22). Cleavage of single-stranded DNA with no ends could occur (22, 23, 26), but it was inhibited by RPA (22). Nuclease activity of Dna2p is maximal under conditions in which Mg^{2+} is high and ATP is low or absent, and the helicase activity is best observed when nuclease activity is lowered by

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§ Present address: Dept. of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Box 712, Rochester, NY 14642. Tel.: 585-275-3269; Fax: 585-275-6007; E-mail: robert_bambara@urmc.rochester.edu.

¹ The abbreviations used are: FEN1, flap endonuclease 1; RPA, replication protein A; ssDNA, single-stranded DNA; SSB, single-stranded binding protein.

TABLE I
Oligonucleotide sequences (5'–3')

Downstream primers ^{a,b}	
D ₁ (60-mer)	TGCGACGTTTC AC GCCTGTTACTTAATTCAGTGGCCGTCGTTTTACAACGACGTGACTGG
D ₂ (43-mer)	CGTACGGACGTAGAGCTGTTTCCAAGTAAAACGACGGCCAGTG
D ₃ (96-mer)	CGACTCTAGAGGATCCCCGGTACCGAGCTCGAATTCGCCCGTTTCACGCCCTGTAGTTAATTCAGTGGCCGT CGTTTTACAACGACGTGACTGGG
D ₄ (76-mer)	GTACCGAGCTCGAATTCGCCCGTTTCACGCCCTGTAGTTAATTCAGTGGCCGTCGTTTTACAACGACGTGACTGGG
D ₅ (100-mer)	GACTCTCGACTCAGTACGCTGTTTTCACGCCCTGTAGTTAATTCAGTGGCCGTCGTTTTACAACGACGTGACTGGG AAACGACGGCCAGTGCTACGAG
D ₆ (58-mer)	TGCG X CGTTTCACGCCTGTTACTTAATTCAGTGGCCGTCGTTTTACAACGACGTGACTGGG
D ₇ (55-mer)	AGGTCTCGACTAACTCTAGTCTGTTTCCACCCGTCACCCGACGCCACCTCCTG
D ₈ (25-mer)	CGACCGTGCCAGCCTAAATTTCAAT
D ₉ (20-mer)	GACGAATTCGGATACGACG
Upstream primers	
U ₁ (26-mer)	CGCCAGGGTTTTCACGTCACGACCA
U ₂ (25-mer)	CGCCAGGGTTTTCACGTCACGAC
U ₃ (26-mer)	CGACCGTGCCAGCCTAAATTTCAATA
U ₄ (18-mer)	CCACCCGTCCACCCGACG
U ₅ (20-mer)	TGCTGGGATCTACAACCAA
Templates	
T ₁ (48-mer)	GCCAGTCACGTCGTTGTAACACGGGTCGTGACTGGGAAAACCCCTGGCG
T ₂ (44-mer)	GCCTGGCCGTCGTTTACGGTCTGACTGGGAAAACCCCTGGCG
T ₃ (49-mer)	GCCAGTCACGTCGTTGTAACACGGGTCGTGACTGGGAAAACCCCTGGCG
T ₄ (76-mer)	GCTCGTAGCACTGGCCGTCGTTTACGGTCTGACTGGGAAAACCCCTGGCGAACAGCTCTACGTGAGTCCA GAGTC
T ₅ (46-mer)	GCCAGTCACGTTGTAACACGGGTCGTGACTGGGAAAACCCCTGGCG
T ₆ (51-mer)	GCAGGAGGTGGCGTCGGGTGGACGGGATTGAAATTTAGGCTGGCACGGTCCG
T ₇ (72-mer)	ATTGAAATTTAGGCTGGCACGGTGGCACCTGGCCGTCGATATCCGAGAGGTGGCGTCGGGTGGACGGGTGG
T ₈ (110-mer)	TTGGTTGTAGGATCCACGACATTGAAGGCAGGAGGTGGCGTCGGGTGGACGGGTGGATTGAAATTTAGGC TGGCACGGTCCGCACTGGCCGTCGTATCCGGAATTCGTC
Blocking primers	
B ₁ (18-mer)	CAGCTCTACGTCCTGACG
B ₂ (20-mer)	CCGGGATCCTCTAGAGTCG
B ₃ (21-mer)	GGCGAATTCGAGCTCGGTAC
B ₄ (23-mer)	ACGGCCAGTGAATTAACCTAACG

^a The boldface nucleotides represent the location of modification. D₆ contains either guanosine or 3-amino-thiocyanato-1,2-propanediol at position X. D₁ contains DNA side chains.

^b The underlined nucleotide indicates a biotin modification.

the presence of high ATP and lower Mg²⁺. One or the other of the two functional activities can be observed preferentially by changing the balance of ATP and Mg²⁺ (10, 23, 25). Unwinding assays show that Dna2p utilizes the 5'-end of single-stranded DNA as an efficient entry point, and this activity is increased as the tail length increases up to 25 nucleotides (25). It was suggested that 3'-nuclease is not relevant for Dna2p because ATP and RPA inhibit this activity (10, 22). Although Dna2p could also bind from the internal single-stranded region prior to displacing the primer segment, this activity is weak and distributive (25). Generally helicases do not exhibit an end preference for substrate binding because a substrate flanked by difference lengths of primers at either end of a template could be utilized to determine the polarity of virtually all the helicases (27). By this criterion, Dna2p is a unique helicase/nuclease (25).

Despite several reports on Dna2p substrate specificity, the mechanism behind the free end preference remains unanswered. We wanted to know the events that occur when Dna2p encounters a flap substrate. We speculated that Dna2p employs a FEN1-like tracking mechanism, because both enzymes share a similar pathway in replication. To test this hypothesis, we utilized substrates capable of defining specific characteristics of the tracking process. Our observations *in vitro* reveal that Dna2p does indeed track on flap substrates. They suggest that this unique mechanism is used to protect against internal cleavage of replication intermediates during Okazaki fragment processing.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Radionucleotides [γ -³²P]ATP (6000 Ci/

mmol) and [α -³²P]dCTP (6000 Ci/mmol) were from PerkinElmer Life Sciences. The T4 polynucleotide kinase (labeling grade), the Klenow fragment of DNA polymerase I, and ATP were from Roche Applied Science. Nuclease-free solutions and reagents were from Ambion, Inc. (Austin, TX). All other reagents were the best available commercial grade.

Enzyme Expression and Purification—*Saccharomyces cerevisiae* Dna2p was cloned into the Sf9 baculovirus expression vector (Invitrogen). The amplification/expression and purification conditions were the same as described previously (23), except that High Five cells were utilized for the final expression step of the protein. *S. cerevisiae* FEN1 was expressed in *Escherichia coli*, and it was subsequently purified under the same conditions described elsewhere (13). *S. cerevisiae* RPA was expressed and purified according to Sibenaller *et al.* (28), except that the Mono-Q column was omitted.

Oligonucleotide Substrates—Oligomer sequences are listed in Table I. Oligomers were annealed as described in the figure legends to form various structures. Modifications to create the branched and amino substrates were as described previously (19). The biotinylated substrates were synthesized by Integrated DNA Technologies (Coralville, IA), and the locations of the biotinylation are indicated as underlined nucleotides in the table. Annealing and labeling conditions were the same as described previously (29), except that the annealing ratio is 1:4:8 (downstream:template:upstream) and 1:2:4 for the reactions containing single-stranded binding protein (SSB) and RPA. Blocking primers were present in 10-fold excess over the annealed flap substrates. All radiolabeled primers were purified by gel isolation from 12 or 15% polyacrylamide, 7 M urea denaturing gels. The 10-nucleotide markers were obtained by 5'-labeling the 10-bp markers from Invitrogen using [γ -³²P]ATP (6000 Ci/mmol) (PerkinElmer Life Sciences), and they were heat-denatured prior to loading of the gel.

Enzyme Assays—Reactions were performed in 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 30 mM NaCl, and various ratios of MgCl₂ and ATP. Enzyme stocks were diluted in 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. The "maximum-nuclease" assays include excess $MgCl_2$ over ATP (2 mM $MgCl_2$ and 1 mM ATP), whereas the "maximum-helicase" assays contain excess ATP over $MgCl_2$ (1 mM $MgCl_2$ and 2 mM ATP). Most assays were performed under one of these conditions. Each reaction contains 5-fmol substrates in a 20- μ l reaction volume with different amounts of the enzymes as indicated in the figure legends. All the assays were preincubated at 37 °C for 5 min (maximum-helicase conditions with substrates, enzyme, and ATP or maximum-nuclease conditions with substrates and enzymes). The reactions were initiated at 37 °C for 10 min with the addition of $MgCl_2$ (for maximum-nuclease conditions, ATP was added with $MgCl_2$ at the end of the preincubation time). Reactions were then stopped by the addition of 20 μ l of 2 \times termination dye (95% formamide (v/v) with bromophenol blue and xylene cyanole). The denatured reactions were resolved on 12 or 15% polyacrylamide, 7 M urea denaturing gels. Each gel was quantitated using a PhosphorImager (Amersham Biosciences) and analyzed using ImageQuant version 1.2 software from Amersham Biosciences. In all studies, the quantitated amount of substrates and products were utilized to calculate the percentage of product formation from the product/(substrate plus product) ratio. The % product formation is indicated below each gel in the figures. This method allows for the correction of any loading errors among lanes. All assays were performed at least in triplicate, and representative assays are shown.

RESULTS

To dissect out the multiple activities of Dna2p, we employed previously developed reaction conditions. One favors maximum-nuclease ($MgCl_2 > ATP$) in which high $MgCl_2$ supports nuclease activity, masking the helicase activity by cutting the flap substrate. The other favors maximum-helicase ($ATP > MgCl_2$ and with a 5-min preincubation of the enzyme and substrate) in which excess ATP sequesters free $MgCl_2$ reducing the nuclease activity and allowing helicase to interact with the substrates (10). By controlling these conditions, we can examine the cleavage properties of Dna2p nuclease and the influence of the helicase activity on Dna2p nuclease on different substrates. These conditions also allow us to monitor the tracking mechanism in the presence and absence of helicase activity with the wild-type protein.

Dna2p Makes Multiple Cuts on a Long Flap—It was shown previously that Dna2p possesses a weak, distributive helicase activity. This is able to influence the positions of cleavage under maximum-helicase conditions, with a flap that is 27 nucleotides or longer (10, 22, 26). We first examined the patterns of nuclease cleavage under nuclease *versus* helicase conditions. Specifically we wanted to know whether Dna2p cleaves a long flap in a progressive fashion by making a series of cuts that shorten the flap in a stepwise manner. To accomplish this, we employed 38-nucleotide flap substrates labeled at either the 3'- or 5'-end of the downstream primer (Fig. 1). Fig. 1, lanes 1–7 and 8–14, shows cleavages of the 5'-radiolabeled and 3'-radiolabeled substrates, respectively, compared under both conditions. 5'-Radiolabeled substrates show that longer cleavage products are released under maximum-helicase conditions (Fig. 1, compare lanes 2–4 to 5–7), similar to what was observed previously (10, 22). This indicates that Dna2p progresses further toward the flap junction before allowing the first cleavage or that inhibiting the nuclease allows further translocation before cleavage. This is also consistent with the earlier report that the nuclease product is longer in the presence of ATP (30). However, most initial cleavages are not close to the base of the flap (38 nucleotides from the 5'-labeled end) under either condition. The downstream duplex was not unwound at all, consistent with other observations that the helicase activity is weak (10, 26).

Examination of the 3'-substrates (Fig. 1, lanes 8–14) shows that majority of the products represent cleavages near the base of the flap. Because the products from the 3'-substrates reflect the sum of all cleavage events occurring on one substrate, the

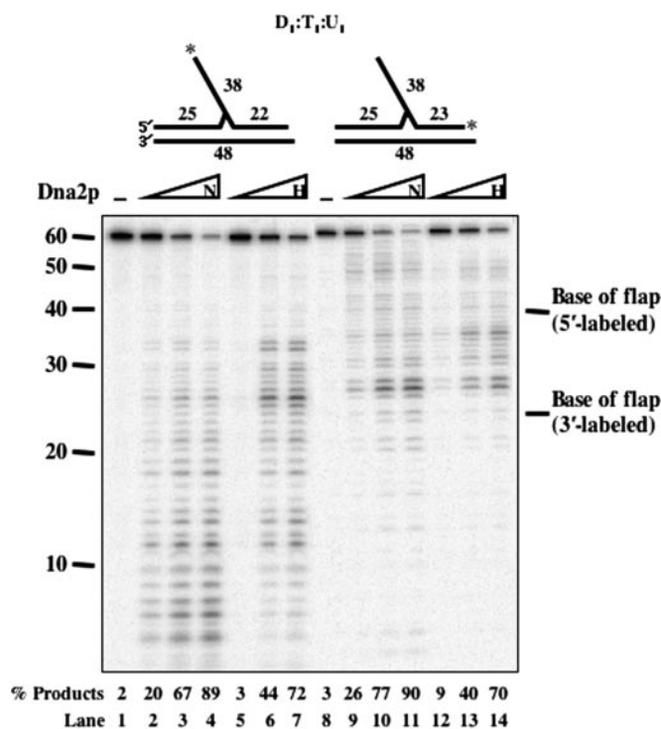


FIG. 1. Dna2p makes multiple cuts on a flap. A Dna2p titration assay (0, 10, 50, and 100 fmol, as indicated by the triangles) under either maximum-nuclease or maximum-helicase conditions was employed. The experimental conditions and the labeling protocols were the same as described under "Experimental Procedures." An extra nucleotide at the 3'-end of the template is purposely designed in all substrates, creating a one-nucleotide overhang, that allows 3'-nucleotide addition by Klenow fragment. Both substrates tested are in 38-nucleotide nicked double flap configuration and contain exact sequences ($D_1:T_1:U_1$). The only difference in these substrates is that one is 5'- ^{32}P -radiolabeled and the other is 3'- ^{32}P -radiolabeled, in lanes 1–7 and lanes 8–14, respectively. The numbers of nucleotides on the flap and numbers of base pairs on the duplex regions are indicated. The 3'-labeled substrate contains one more nucleotide in the downstream annealed duplex, creating a blunt end (23 nucleotides annealed on the downstream duplex), because of the addition of the radioactive dC. In the 5'-labeled substrate, the one-nucleotide overhang is represented as a staggered end in the schematic diagram. Lanes 1 and 8 are the substrate-only controls. Lanes 2–4 and 9–11 are under maximum-nuclease conditions, whereas lanes 5–7 and 12–14 are under maximum-helicase conditions. Schematic representations of the substrate structures are depicted on the top of the gel, and a nucleotide length marker series is indicated at the left side of the gel. Percent product formation, defined as "(product/(product + starting substrates)) \times 100," is indicated at the bottom of the gel. N, maximum-nuclease; H, maximum-helicase.

fact that these are distributed closer to the flap base under both conditions implies that each substrate sustained multiple progressive cuts by the nuclease. These results show progression but do not define whether the cleavage process is processive, *i.e.* carried out by the same protein. Several Dna2p proteins may collaborate to reduce the flap length.

Dna2p Cleavage Requires a Free 5' Terminus—Dna2p was found to prefer free 5'-ends (10, 22, 25), and primers annealed to a free ssDNA and biotin-streptavidin conjugation can decrease Dna2p nuclease activity (22). We asked whether the end preference was influenced by the helicase function because the helicase had been reported to allow Dna2p to load internally, and Dna2p was shown to cleave some substrates in which end loading was not possible (23, 25, 26). We tested substrates that contain a downstream primer with a 25-nucleotide flap (Fig. 2A). On one substrate a 17-nucleotide primer was annealed at the 5'-end of the flap (Fig. 2A, lanes 8–14). Under both maximal-nuclease and maximal-helicase conditions, Dna2p was able to cleave the 25-nucleotide flap (Fig. 2A, lanes 1–7). How-

function does not allow the bypass of the 5'-end requirement for Dna2p loading onto a substrate. A larger gap between the blocking primer and the flap base (53 nucleotides) also shows similar results as described below (Fig. 3A), ruling out the possibility that the eight nucleotides is too short for effective Dna2p binding. Apparently 5'-end loading is also a requirement for cleavage on these substrates even when the helicase is maximally active (Fig. 3A, compare lanes 5–7 to 12–14).

A bubble substrate is defined as having the 5'-end of the flap annealed to the template. Such substrates resist FEN1 cleavage because of its 5'-end requirement (20, 29). The bubble structure creates a particularly convincing substrate for testing the requirement for a 5'-end. Primers annealed to flaps allow some FEN1 access presumably because annealing is not 100% efficient (data not shown). However, the two-site annealing of the bubble primer appears to make it a stable configuration. Bubble substrates were tested with Dna2p (Fig. 2B). Alternative structures were created that simulate Okazaki fragment intermediates either with no upstream primer, a fully annealed upstream primer, or an upstream primer that forms a double flap. In all three cases the single-stranded bubble region is long enough to ensure space for enzyme binding (50 nucleotides). Even though a 53-nucleotide control flap substrate sustained expected cleavages under both conditions (Fig. 2B, lanes 1–7), Dna2p failed to cleave any of the bubble structures (lanes 8–28), supporting an absolute requirement for Dna2p to load at a 5'-end. This result is fully consistent with the blocking effect of a primer annealed on the flap and indicates that Dna2p is not designed for internal access to single-stranded DNA.

Dna2p Cleavage Employs a Tracking Mechanism—The 5'-end requirement is consistent with two alternative mechanisms. One is a tracking mechanism in which the enzyme enters from the 5'-end and then traverses the single-stranded region to the point of cleavage. The other is a looping mechanism. In this scenario, binding to the 5'-end of the flap activates the enzyme for subsequent functions. It is then able to bind the single-stranded region for movement or cleavage by looping the flap so that the 5'-end is near the site of cleavage. During this process the enzyme is bound at two sites on the flap. To delineate these two mechanisms, we first employed substrates that were used to define the tracking mechanism of FEN1 (14) (Fig. 3). A 73-nucleotide flap substrate was constructed, and an oligomer was annealed to the flap at its 5'-end, in the middle, or at its base (Fig. 3A). We reasoned that if Dna2p employs a looping mechanism, the substrate with the primer in the middle of the flap would be cleaved in the 32-nucleotide gap region between the primer and the flap base. This is because the Dna2p can access both the 5'-end and a cleavable internal region. The control substrate was cleaved well by Dna2p under both conditions (Fig. 3A, lanes 1–7). As expected, the 5'-terminal blocking primer inhibited Dna2p cleavage (Fig. 3A, lanes 8–14). We note that the 53-nucleotide region provides a large area of access with a variety of sequences and structures, and yet virtually no cleavage is seen (Fig. 3A, lanes 8–14). With the flap containing a primer in the middle (Fig. 3A, lanes 15–21), the 32-nucleotide gap is not cleaved. Instead, substantial cleavage is observed in the 20-nucleotide region between the 5'-end and the primer. This result argues against the looping mechanism. It indicates that Dna2p loads onto the 5'-end and tracks to the point where it is blocked by the primer. Results with the primer placed at the flap base reinforce this conclusion (Fig. 3A, lanes 22–28). In that case, Dna2p appears to track the further distance to the primer, cleaving throughout the single-stranded region. Effective blockage by the primer is consistent with the notion that

Dna2p possesses a weak helicase activity, incapable of displacing a 23-nucleotide primer (10, 23, 25, 26). Note that Dna2p cleaves right up to the site of the block (Fig. 3A), whereas it only gets to within 5–7 nucleotides of the flap junction on other substrates. Perhaps the fork structure sterically hinders further cleavages closer to the flap junctions.

In another approach to distinguish a tracking from a looping mechanism, we placed biotin modifications either at the 5'-end or in the middle of 53-nucleotide flaps (Fig. 3B). The unmodified flaps are cleaved equally well by Dna2p in the absence or presence of streptavidin (Fig. 3B, lanes 1–6), indicating that unbound streptavidin does not interfere with Dna2p catalysis. A substrate biotinylated at the 5'-end is cleaved by Dna2p in the absence of streptavidin (Fig. 3B, lanes 7–9), implying that the biotin modification does not inhibit the recognition of the 5' terminus of DNA, a result obtained previously (22). Conjugation of streptavidin to the biotinylated substrate inhibits cleavage (Fig. 3B, lanes 10–12), presumably by blocking the entry of Dna2p. This is consistent with the results obtained from the blocking primers used above. When streptavidin is conjugated to a biotin in the middle of the flap (Fig. 3B, lanes 16–18), cleavage occurs prior to the conjugation site but not beyond it. In principle, this substrate is similar to the substrate with a primer in the middle of the flap. In fact, the biotin-streptavidin flap should be even more flexible than the primed flap to allow for looping. The absence of cleavage beyond the conjugation site is additional evidence that the 5'-end is an entry site for tracking and not an activation site for looping. This result also verifies that the helicase function of Dna2p cannot force the dissociation of the streptavidin.

Dna2p Can Track Over Alternative Structures and Does Not Require Contact with a Continuous Series of Nucleotides—Because cleavages by Dna2p occur on biotinylated flaps in the absence of streptavidin (Fig. 3B, lanes 13–15), Dna2p must be able to track over a bulky adduct on DNA. This suggests that tracking does not require Dna2p contact with a series of unmodified nucleotides. To assess further the nucleotide structure requirement of the flap for tracking, we tested a flap in which two adjacent nucleotides were separated by a 3-aminothiocyano-1,2-propanediol (19) (Fig. 4A). Both the unmodified (Fig. 4A, lanes 1–7) and modified substrates (lanes 8–14) were examined under both maximum-nuclease and maximum-helicase conditions. Dna2p was similarly capable of tracking and cleaving both substrates. This suggests that similar to FEN1, the tracking of Dna2p does not require the contact of a perfect series of natural nucleotide structures on the flap.

The Mechanism Appears to Involve Threading—As discussed above, the tracking mechanism of FEN1 is very forgiving of large structures on the flap including polymer branches (19), indicating that the flap is not threaded through a hole in the protein. To consider this same issue with Dna2p, we employed a substrate with a branched flap (19) (Fig. 4B). The substrate has a 38-nucleotide flap. The 27th residue from the base of the flap is a ribonucleotide with an 11-nucleotide branch attached at the 2'-position. The branch is attached at its 3'-end and has a native free 5'-end. When the unbranched control substrate was tested under both conditions, it was readily cleaved by Dna2p (Fig. 4B, lanes 3–8). It was also susceptible to FEN1 (Fig. 4B, lane 2). The branched substrate was also effectively cleaved by FEN1 (Fig. 4B, lane 10), which evidently tracks over the branch point. However, the cleavage pattern for Dna2p (Fig. 4B, lanes 11–16) suggests tracking from one or the other 5'-end only up to the branch point. Cleavages were present from the 5'-end to the branch point. No cleavages were observed after the branched point, suggesting that Dna2p exhibits a "threading-like" mechanism. Presumably, the hole or cleft

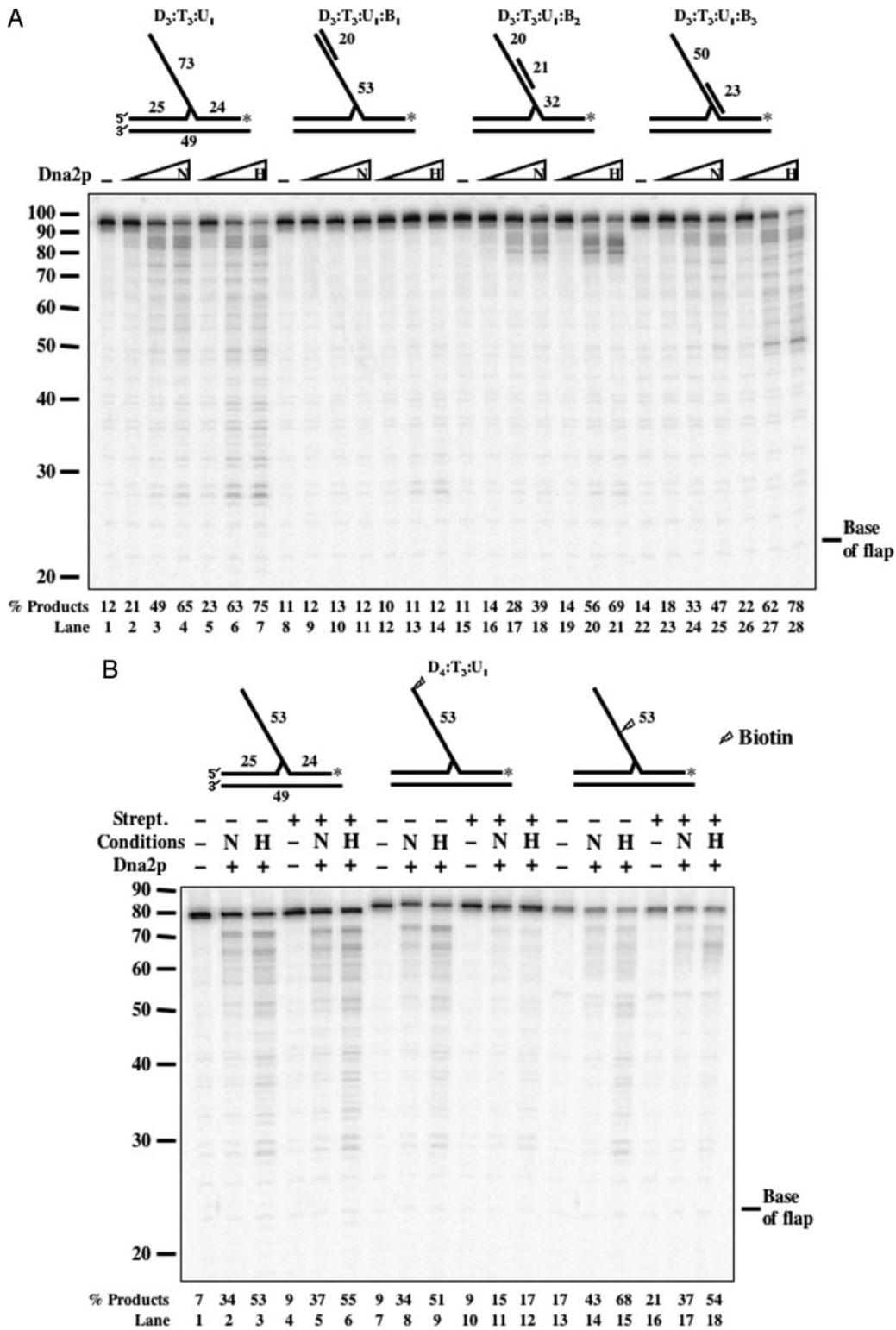


FIG. 3. Cleavage by Dna2p requires a tracking mechanism. *A*, Dna2p titration assay (0, 10, 50, and 100 fmol, as indicated by the triangles) under either maximum-nuclease or maximum-helicase conditions was employed. The assay conditions were the same as described under "Experimental Procedures." Lanes 1–7 contain 3'-radiolabeled 73-nucleotide double flap substrates ($D_3:T_3:U_1$) and a poly(dT)₁₆ primer, a non-complementary primer, as the no-blocking primer control. Lanes 8–14, 15–21, and 22–28 contain blocking primers B₁, B₂, and B₃, respectively, that are annealed at distances leaving a gap of 53, 32, and 0 nucleotides from the first annealed nucleotide on the downstream duplex DNA. Lanes 1, 8, 15, and 22 are the substrate-only controls. Lanes 2–4, 9–11, 16–18, and 23–25 are under maximum-nuclease conditions, whereas lanes 5–7, 12–14, 19–21, and 26–28 are under maximum-helicase conditions. Schematic representations of the substrate structures are depicted on the top of the gel. For *A* and *B*, the numbers of nucleotides in the flap and numbers of the base pairs in the duplex regions are labeled. Percent product formation is indicated at the bottom of the gel. A 10-nucleotide length marker series is indicated at the left side of the gel. *B*, a single amount of Dna2p (50 fmol) was utilized in each reaction. Substrates (5 fmol) were preincubated with and without 25-fold excess of streptavidin for 5 min at 37 °C and followed by either maximum-nuclease (lanes 2, 5, 8, 11, 14, and 17) or maximum-helicase (lanes 3, 6, 9, 12, 15, and 18) conditions as described under the "Experimental Procedures." Lanes 1–6 contain the non-biotinylated substrate control ($D_4:T_3:U_1$). Lanes 7–12 and 13–18 include substrates ($D_4:T_3:U_1$) that are biotinylated at the 1st or the 25th nucleotide from the 5'-end of the flap, respectively. The experimental setup and the schematic representation of the substrates are indicated at the top of the gel, and a 10-nucleotide length marker series is indicated at the left side of the gel. *N*, maximum-nuclease; *H*, maximum-helicase.

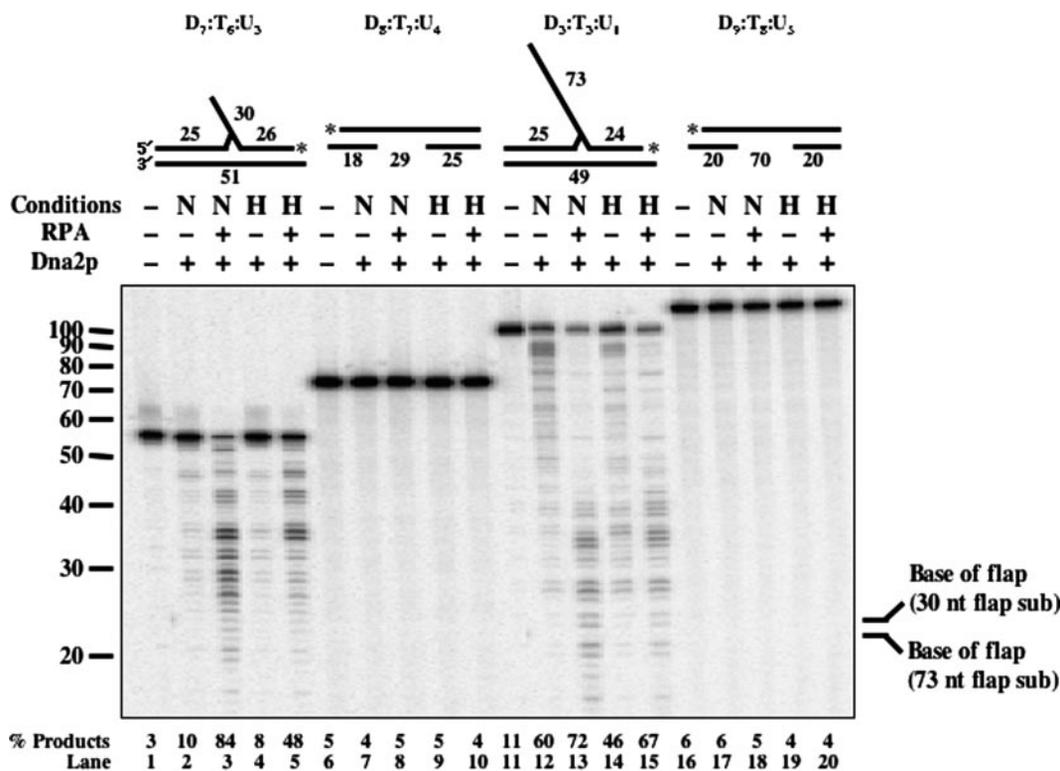


FIG. 5. The tracking mechanism prevents Dna2p from cleaving replication intermediates. A reaction with Dna2p (100 fmol) and RPA (100 fmol) was performed under maximum-nuclease (lanes 2–3, 7–8, 12–13, and 17–18) or maximum-helicase (lanes 4–5, 9–10, 14–15, and 19–20) conditions, as described under “Experimental Procedures.” A 3′-radiolabeled 30-nucleotide (D₇:T₆:U₃) and a 3′-radiolabeled 73-nucleotide (D₃:T₃:U₁) double flap control reactions were done in lanes 1–5 and 11–15, respectively. A 5′-radiolabeled 29-nucleotide (D₅:T₇:U₄) and a 5′-radiolabeled 70-nucleotide (D₉:T₈:U₅) gap substrates mimicking replication intermediates were tested in lanes 6–10 and 16–20. Lanes 1, 6, 11, and 16 are the substrate-only controls. Schematic representations of the substrate structures are depicted on the top of the gel, and a nucleotide length marker series is indicated at the left side of the gel. The numbers of the nucleotides in the flap and numbers of the base pairs in the duplex regions are labeled. Percent product formation is indicated at the bottom of the gel. N, maximum-nuclease; H, maximum-helicase.

The Tracking Requirement of Dna2p Should Protect Replication Intermediates—Dna2p is a single strand DNA-specific endonuclease, and there have been reports indicating that it cleaves internally on a stretch of single-stranded DNA or a single-stranded circular plasmid, although this activity is weak (22, 23, 26). This internal binding property would allow Dna2p to bypass the tracking mechanism and potentially cleave the single-stranded regions between Okazaki fragments. To examine possible accessing of substrate without tracking, we designed different lengths of gap substrates flanked by two primers, and we tested Dna2p cleavage on these substrates under both maximum-nuclease and maximum-helicase conditions and in the presence and absence of RPA (Fig. 5). Contrary to previous results, we found that Dna2p is not able to cleave single-stranded DNA internally (Fig. 5, lanes 6–10 and 16–20). The gaps of either 29 or 70 nucleotides are not cleaved, whereas the control substrates with flaps either 30 or 73 nucleotides long continue to exhibit characteristic cleavage patterns by Dna2p (Fig. 5, lanes 1–5 and 11–15). RPA did not allow Dna2p to enter the substrate without a free single-stranded end (Fig. 5, lanes 8, 10, 18, and 20), suggesting that the RPA-mediated stimulation mechanism does not bypass tracking (31). The presence of RPA sometimes alters the cleavage patterns by Dna2p (Fig. 5, compare lane 2 to 3, lane 12 to 13, and lane 14 to 15), as observed previously (10). We postulate that as with FEN1 (14), the tracking requirement of Dna2p is designed to prevent the nuclease from accessing the replication intermediates that may cause double strand breaks during replication.

DISCUSSION

Removal of the RNA primers of Okazaki fragments requires formation and cleavage of 5′ single-stranded flaps. At least

some of these are expected to become sufficiently long to require the sequential nuclease actions of Dna2p and FEN1 (6, 8, 10). Considerable evidence supports a mechanism by which FEN1 enters the flap at its 5′-end and then tracks to the base of the flap for cleavage (14, 19, 32). The nuclease and helicase activities of Dna2p have been reported to prefer substrates containing 5′-ends (25, 26). These observations suggested to us that Dna2p is also a tracking enzyme. Here we have analyzed cleavage specificity of Dna2p by using substrates designed to define its tracking characteristics. Results of our analyses uniformly support the conclusion that Dna2p nuclease moves for cleavage by a tracking mechanism similar to that of FEN1. This mechanism is not influenced substantially by the presence of helicase activity.

Dna2p is a helicase with conserved helicase motifs and shows evidence of effective ATP hydrolysis when ssDNA and ATP are both present (25). Under all the conditions tested, the helicase activity is most active when ATP is present in excess over MgCl₂ (10). In fact, the helicase function of Dna2p is essential (9), and the deficient mutant only grows on media containing galactose that supports slow growth (33). However, the helicase function is not robust, and it is not known whether its essential property is manifested in Okazaki fragment processing. Previous studies have shown that the helicase activity aids the processing of initiator primers on long flaps (10, 25). However, our coupled nuclease-helicase assays indicate that the helicase is not effective enough to move down the flap to its base and then displace the annealed regions.

Most DNA helicases bind single-stranded DNA internally and then exhibit ATP-driven motion on that strand, dissociating adjacent annealed strands. Blocking primers on the 5′

terminus of the flap substrates were able to inhibit Dna2p cleavage even though a long gap was presented to Dna2p, which would create enough space for the enzyme to bind directly from the solution (Fig. 3A). Bubble substrates also prevent Dna2p cleavage because the 5'-end of the flap is annealed to the template (Fig. 2B). These results show that cleavage by Dna2p requires entry from the 5' terminus of DNA, not typical of other helicases.

Previously, it was shown that Dna2 nuclease could cleave a single-stranded circular substrate, indicating internal binding (23). However, evidence suggests that this mode of cleavage is not biologically relevant. It is inhibited by the presence of RPA when tested on a single-stranded DNA with flanking double-stranded regions (22). Studies of helicase function of Dna2p revealed that binding is greatly favored on substrates with free 5'-ends because the enzyme could use the 5'-end as an efficient entry point. When the enzyme was given the choice of unwinding a primer by binding the adjacent single-stranded region internally or entering from the end, the enzyme preferred the DNA end (25).

In all the substrates we tested here, we observed an end requirement for Dna2p cleavage. Unlike results reported previously (22, 26), we did not observe internal cleavages with single-stranded regions flanked by primers (Fig. 5) in the presence or absence of RPA. We suspect that *in vivo* Dna2p had evolved to require end entry for the same reason proposed for FEN1 (14). Numerous single-stranded regions are exposed during replication, which, if cleaved, would make double strand breaks in the chromosome. Production of a number of double strand breaks that are not repaired will eventually lead to cell cycle arrest (34).

Results with primers or biotin-streptavidin conjugations placed centrally on a long flap reveal that Dna2p does not employ a looping mechanism, in which binding the 5' terminus activates the protein for second-site binding internal to the single strand (Fig. 3). The tracking process appears to require protein movement down the strand. Additional results show that Dna2p could track over a biotinylated substrate, and a substrate in which one nucleotide was substituted with 3-amino-thiocyano-1,2-propanediol (Figs. 3B and 4A). This means that movement does not require the contact with a series of adjacent unmodified nucleotides. In this way, it is different from replicative DNA polymerase movement on DNA templates, which is inhibited by damaged nucleotides. Most helicases, upon encountering a damaged nucleotide, might be expected to dissociate. Indeed, a benzo[a]pyrene-DNA adduct inhibits the translocation activity of a helicase encoded by gene 4 in bacteriophage T7 (35). A recent study with the human Werner syndrome helicase, a RecQ helicase, and benzo[c]phenanthrene diol epoxide dA adducts also revealed that these helicases are affected by the strand, orientation, and stereochemistry of the adducts (36). However, because of the 5'-entry requirement, Dna2p must be able to traverse the site without dissociation.

The tracking function may be only partly related to the helicase. A 5'-end requirement is observed not only under conditions favoring the nuclease, but even when ATP is totally absent from the reaction (data not shown). Unlike FEN1, Dna2p is able to cleave after a branched point on a modified flap (Fig. 4B), indicating that the strand must fit through a limited sized chamber in the enzyme. This suggests that the tracking process involves an actual threading of the strand through the protein. Whether or not the strand is fully encircled awaits evidence from future crystallographic analyses.

The inhibition of Dna2p cleavage by the prokaryotic SSB is also consistent with a requirement for tracking (21) (data not

shown). SSB is expected to block Dna2p movement on the flap and could also be blocking access to the DNA for cleavage. Inhibition by SSB contrasts with the stimulatory effect of RPA, which must facilitate tracking, cleavage, or both. It is difficult to envision how bound RPA could facilitate threading movement or even allow access for cleavage. It was reported that RPA and Dna2p interact with each other by specific protein-protein contacts via their C termini (31). The fact that Dna2p and RPA interaction requires the formation of a ternary complex with single-stranded DNA and that it affects catalysis raises the possibility that RPA binding to DNA allows the direct recruiting of Dna2p to the site of cleavage, bypassing the rate-limiting tracking step (21). Our preliminary results do not favor this mechanism (Fig. 5); however, further study is needed. Interaction of Dna2p and RPA in the presence of various substrates, using both catalytic and binding assays, should be analyzed. Future detailed examinations of RPA effects on both the helicase and ATPase activities of Dna2p will permit more understanding of the underlying mechanisms of this interaction. Analysis of the interaction of the two proteins by structural approaches will also reveal valuable information on the functions of this protein-protein contact. The very fact that the stimulation was not anticipated, based on the expected inhibitory effect of bound protein, suggests that it is biologically relevant.

RPA-coated flaps are inhibitory to FEN1 tracking (21). The specific stimulation of RPA on Dna2p and the inhibition by RPA of FEN1 tracking and cleavage led to the conclusion that RPA orders the actions of the two nucleases during long flap processing (6, 10, 21).

Overall, our results led us to propose a novel mechanistic model for Dna2p cleavage. Dna2p, like FEN1, will recognize the 5'-end of a flap and track along the single-stranded region prior to cleavage. We summarize the properties of the two tracking enzymes participating in DNA replication below (14, 19, 32). The tracking properties of both nucleases are similar except that Dna2p may employ a threading-like mechanism, whereas FEN1 does not. As suggested before, Dna2p prefers long flaps, especially when they are coated by RPA, whereas FEN1 prefers short flaps as its substrates (6, 10, 21, 23). Only FEN1 can cut at the base of a flap in one cleavage reaction to create the substrate for DNA ligase, although multiple Dna2p cleavages on one substrate may achieve similar results *in vitro* (6, 21). This property also supports the proposed order of action for the two nucleases (10, 21).

The nuclease activity of FEN1 alone has been proposed to be sufficient for processing most of the flaps in Okazaki fragment maturation (6, 10). Unprocessed flaps are the intermediates leading to recombination and repeat sequence expansion (37–40). The null mutant of FEN1 in *S. cerevisiae*, *rad27Δ*, exhibits a hyper-recombinogenic phenotype and a many-fold increase in genomic instability (29), whereas *dna2-1* mutants only show 2–3-fold increase in both dinucleotide instability and trinucleotide fragility (CAG-155 tract) (41, 42). This indirectly reveals that FEN1 has a broader role in processing flaps than Dna2p during replication.

Structure-containing flaps inhibit both Dna2p and FEN1 cleavages (10, 20). An exception is a flap with a foldback, also with an unstructured 5'-end region, which allows Dna2p access under conditions in which the helicase is active (10, 25). This suggests that Dna2p may be designed to process only a subset of structured flaps. Other factors may be involved to work with Dna2p to process most of the structured flaps (10, 22). Possible candidates are Rrm3p, Pif1p, and Sgs1p, the yeast counterpart of BLM and WRN (43–45).

The tracking mechanism of Dna2p may also have other roles

in cellular DNA metabolism, because processing of Okazaki fragments may be only one of many biological functions of Dna2p (6, 10). The essential nature of Dna2p in DNA metabolism is under intensive study. Many studies have revealed that it may be part of the double strand break repair pathway or ribosomal DNA and telomere replication (44, 46, 47). Clearly, further study of the interaction of Dna2p with other proteins for cleavage of long flaps is necessary just to understand its role in DNA replication.

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