

# Dna2 is a structure-specific nuclease, with affinity for 5'-flap intermediates

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## ABSTRACT

**Dna2 is a nuclease/helicase with proposed roles in DNA replication, double-strand break repair and telomere maintenance. For each role Dna2 is proposed to process DNA substrates with a 5'-flap. To date, however, Dna2 has not revealed a preference for binding or cleavage of flaps over single-stranded DNA. Using DNA binding competition assays we found that Dna2 has substrate structure specificity. The nuclease displayed a strong preference for binding substrates with a 5'-flap or some variations of flap structure. Further analysis revealed that Dna2 recognized and bound both the single-stranded flap and portions of the duplex region immediately downstream of the flap. A model is proposed in which Dna2 first binds to a flap base, and then the flap threads through the protein with periodic cleavage, to a terminal flap length of ~5 nt. This resembles the mechanism of flap endonuclease 1, consistent with cooperation of these two proteins in flap processing.**

## INTRODUCTION

Genomic instability has many adverse cellular consequences, producing genetic disorders and predisposing to cancer (1). Proper DNA replication and repair are key to stabilizing the genome. Successful DNA replication duplicates the DNA in an error-free manner (2). However, minor errors occur and are compounded by internal and external DNA-damaging events. These errors and events necessitate multiple DNA repair pathways. Both DNA replication and repair involve the coordination of multi-subunit protein complexes. A number of the protein components involved in DNA replication are shared by DNA repair pathways. One such protein is Dna2 (3–7).

Dna2 enzymatic functions are conserved from yeast to humans (8,9). It possesses a variety of activities, including single-stranded (ss)DNA endonuclease (10,11), 5' to 3' ATP-dependent helicase (12,13), strand annealing (14) and strand exchange (14). Both nuclease and helicase functions are essential for cell viability in *Saccharomyces cerevisiae* (3,11,15).

During DNA replication, Dna2 is proposed to function in processing the RNA/DNA primer, which initiates Okazaki fragments. For processing, the primer is displaced into a 5'-flap intermediate. Two pathways are proposed to work in parallel to resolve all Okazaki fragments. One pathway involves the creation of short flaps, which are cleaved by flap endonuclease 1 (FEN1) (16,17). FEN1 is a structure-specific endonuclease that recognizes and cleaves 5'-flap intermediates to create a nick (18). Both Dna2 and FEN1 binding and cleavage occur independent of DNA sequence (11,13,19–25). After FEN1 cleavage, DNA ligase I joins the upstream and downstream Okazaki fragments. A second pathway involves FEN1, Dna2 and the single-stranded binding protein, replication protein A (RPA) (21,24). Dna2 interacts with both FEN1 and RPA (21,26). In this pathway, long flap intermediates are bound by RPA, which inhibits FEN1 cleavage. Dna2 is able to resolve these flaps by displacing the flap-bound RPA and cleaving to create a short, 4–6 nt, RPA-free flap (25). FEN1 then removes Dna2 from the short flap and cleaves to create a nick for ligation (23,24).

Dna2 is also proposed to participate in DNA repair pathways. In double-strand break (DSB) repair, studies suggest that Dna2, along with the helicase Sgs1, is involved in resection of the 5' strand after initial processing by the MRX (Mre11/Rad50/Xrs2) complex (7,27,28). In addition, human Dna2 was shown to localize to both the nucleus and mitochondria, where it is proposed to be involved in both nuclear and mitochondrial DNA replication and repair (6,9,29). One study suggests that Dna2 participates in mitochondrial long-patch base excision

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**Table 1.** Oligonucleotides sequences

Oligo	Length (nt)	Sequence (5'–3')
Flap	98	TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTC GCG AAC CCT ATT TAG GGT TCG CG
ssDNA	30	TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT
Fork	87	TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTT GTT ACC CTT TGA
Double-Flap	99	TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTC GCG AAC CCT ATT TAG GGT TCG CGT
5'-Tail	74	TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GT
Gap	88	ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTT TTT TTT TTT TTT TTT TTT TCG CGA ACC CTA TTT AGG GTT CGC G
Nick	68	ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTC GCG AAC CCT ATT TAG GGT TCG CG
Biotin-Flap	98	/Biotin/TTC ACG AGA TTT ACT TAT TTC ACT GCG GCT ACA TGA TGC ATC GTT AGG CGA TTC CGC CTA ACG ATG CAT CAT GTC GCG AAC CCT ATT TAG GGT TCG CG

repair (LP-BER), and if so is likely to support that same mode of DNA repair in the nucleus (6). Furthermore, Dna2 may also play a role in chromatin dynamics (4).

Previous studies demonstrated that Dna2 cleaves a variety of ssDNA substrates, with a pattern of cuts suggesting entry from the 5'-end and movement in the 5' to 3' direction (10,11,13,30). Both Dna2 and FEN1 require an unobstructed 5'-end for cleavage (30,31). Cleavage of substrates with a free 5' single-stranded end by Dna2 occurs with similar efficiency regardless of the single or double-stranded structure in other parts of the substrate. Yet, despite these findings, Dna2 participates in a number of pathways in which it cleaves within single-stranded regions of substrates that form flaps or 5' single-strand extensions from a double strand. These observations led us investigate whether Dna2 requires a specific DNA structure for optimal cleavage, similar to that required by FEN1. We show here that Dna2 recognizes and binds specific structural features of the DNA. These findings are consistent with cellular DNA intermediates that require processing by Dna2, and define Dna2 as a structure-specific nuclease.

## MATERIALS AND METHODS

### Oligonucleotide substrates

All substrates used in this study are listed in Table 1. Synthetic oligonucleotide substrates, including the ones with biotin modifications, were purchased from Integrated DNA Technologies. The substrates were designed to fold into the desired conformation or remain in single-stranded form, as stated in the figure legends. The conformations represented are based on the lowest free energy predictions, which were obtained from *mfold* (32,33). Substrates were labelled at the 5'-terminus with polynucleotide kinase (Roche) as described earlier (23). The substrates were then purified by denaturing

**Table 2.** Relative Dna2 E675A binding constants

Substrate	$K_d$ (nM) <sup>a</sup>
Flap	8.4 ± 2.7
ssDNA	29 ± 9.1
Fork	9.2 ± 2.5
Double Flap	7.0 ± 2.5
5'-Tail	8.5 ± 2.5
Gap	6.7 ± 2.5
Nick	54 ± 9.1

<sup>a</sup>Value are an average of three independent experiments. Error values indicate ±SEM

polyacrylamide gel electrophoresis (PAGE) and resuspended in 1× TE.

### Recombinant Dna2

Dna2 E675A was produced by site-directed mutagenesis as described (11). Wild-type Dna2 and Dna2 E675A were over-expressed in baculovirus High Five cells. The proteins were then affinity purified as described earlier (11).

### Gel shift assay

The reaction buffer contained 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 0.1 mg/ml BSA, 2 mM dithiothreitol, 5% glycerol, 2 mM MgCl<sub>2</sub> and 1 mM adenosine triphosphate (ATP) in a volume of 20 μl. In Figure 1D and E and Table 2, increasing amounts of Dna2 E675A were incubated with 5 fmol of labelled substrates at room temperature for 10 min. In Figures, 2A and B, 3B and 4A, increasing amounts of Dna2 E675A were incubated with a mixture of 5 fmol of radiolabelled and 1 pmol of unlabelled substrate, as indicated in the figure legends. In Figure 4A, streptavidin (5 pmol) was incubated with the biotin-flap substrate (1 pmol) for 10 min at 37°C prior to the addition of Dna2. Upon the addition of Dna2 E675A, the reactions were incubated at room temperature for 10 min. DNA-protein complexes were then

resolved on a pre-run 6% TBE gel (Invitrogen), which was then subjected to electrophoresis at 150 V for 30–40 min.

### Nuclease assay

The reaction buffer contained 50 mM Tris–HCl, pH 8.0, 30 mM NaCl, 0.1 mg/ml BSA, 2 mM dithiothreitol, 5% glycerol, 2 mM MgCl<sub>2</sub> and 1 mM ATP in a volume of 20  $\mu$ l. In Figures 1B and C, and 3A, increasing amounts of Dna2 were incubated with 5 fmol of labelled substrates for 10 min at 37°C, as indicated in the figure legends. In Figures 2C and D, 3C and 4B, reactions contained 5 fmol of radiolabelled substrate, 1 pmol of unlabelled substrate and 50 fmol of wild-type Dna2. Prior to the addition of Dna2, the labelled and unlabelled substrates were mixed. In Figure 4B, streptavidin (5 pmol) was incubated with the biotin-flap substrate (1 pmol) for 10 min at 37°C prior to the addition of Dna2. Upon addition of Dna2, reactions were incubated at 37°C. Aliquots were taken at different times, and the reaction stopped in each by the addition of 20  $\mu$ l of 2 $\times$  termination dye (90% v/v formamide, 0.01% bromophenol blue, and 0.01% xylene cyanole). Reactions were then heated to 95°C for 5 min and analyzed on a 15% polyacrylamide/7 M urea gel, which was subjected to electrophoresis at 80 W for 1–1.5 h.

### DNase I footprinting assay

The reaction buffer contained 50 mM Tris–HCl, pH 8.0, 30 mM NaCl, 0.1 mg/ml BSA, 2 mM dithiothreitol and 5% glycerol in a volume of 20  $\mu$ l. Dna2 E675A was incubated with 10 fmol of a flap substrate for 10 min at room temperature. The reactions were then subjected to footprinting analysis. A mixture of 40 mM MgCl<sub>2</sub>/10 mM CaCl<sub>2</sub> (3  $\mu$ l) was added to the reaction for 2 min at room temperature followed by the addition of 3  $\mu$ l of DNase I (0.1 U/ $\mu$ l) (Promega) for 2 min at room temperature. The reactions were then stopped by the addition 15  $\mu$ l of stop solution (10 mM EGTA, pH 8.0, 45% v/v formamide, 0.01% bromophenol blue and 0.01% xylene cyanole) and incubated at 95°C for 5 min prior to loading. They were then analyzed on a 15% polyacrylamide/7 M urea gel, which was subjected to electrophoresis at 80 W for 1–1.5 h.

### Gel analysis and quantitation

At least three independent experiments were performed for each figure and representative gels are shown. After running conditions, the gels were dried on a gel dryer with vacuum. The dried gels were then exposed to a phosphor screen and visualized by phosphor imaging (GE Healthcare). The images were then analyzed and quantitated using ImageQuant software. The markers shown in Figures 2C and 5 are based on the migration of a radiolabelled 10-bp ladder (Invitrogen). Graphs are an average of at least three independent experiments. Bars represent the standard deviation. Percent bound is defined as (bound/(bound + unbound))  $\times$  100. Percent cleavage is defined as (cleaved/(cleaved + uncleaved))  $\times$  100. Relative Dna2 binding was normalized to the percent bound with 400 fmol of Dna2 E675A on the flap

alone. Relative Dna2 cleavage was normalized to the percent cleavage by Dna2 at 10 min on the flap alone.

### Calculation of dissociation constants

After gel shift analysis, curves were fit using non-linear least squares regression of the hyperbolic equation,  $y = B_{\max} \times [\text{Protein}] / (K_d + [\text{Protein}])$ , where  $y$  is the percent of oligonucleotide bound,  $[\text{Protein}]$  is the concentration of protein in nanomoles,  $B_{\max}$  is the maximum binding, and  $K_d$  is the equilibrium dissociation constant.

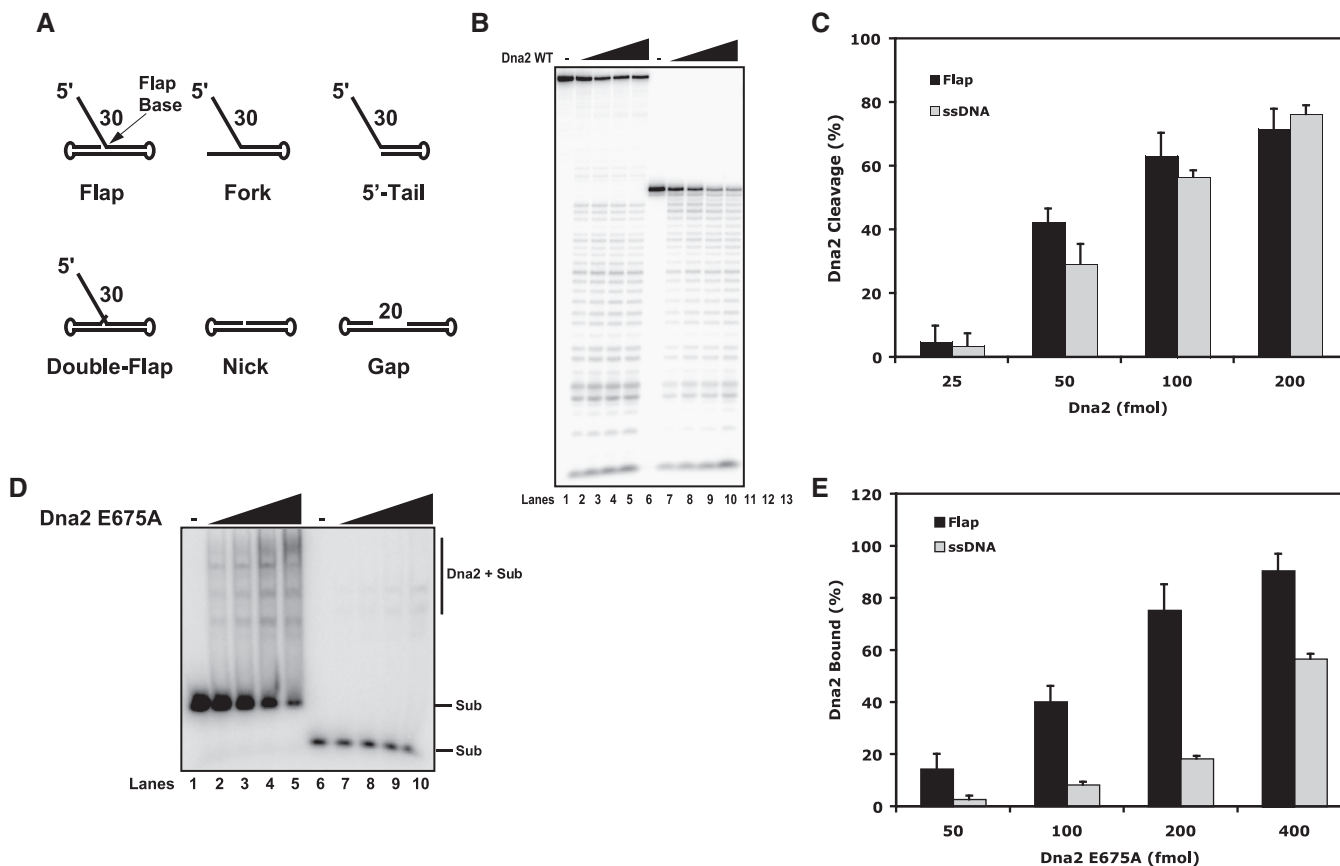
## RESULTS

### Oligonucleotide substrate design

The ssDNA and 5'-flap substrates were designed such that the 30-nt ssDNA substrate was the exact sequence as the 30-nt ssDNA portion of the flap substrate, which allowed for the direct comparison of the utilization of each substrate by Dna2, independent of sequence (Table 1). Furthermore, the ssDNA sequence was constructed to prevent the formation of DNA secondary structure, so that it would represent a genuine single strand, and not fold into a flap-like configuration. We also designed the flap substrate as a single oligonucleotide, which folded into the desired flap configuration, allowing the creation of a genuine flap base (34). The flap base is defined as the junction between the duplex portion of the downstream segment and the single-stranded flap region (Figure 1A). This was done by employing a hairpin design that allowed annealing of the upstream and downstream regions of the flap. By utilizing a single oligonucleotide, we eliminated the need to create the flap by annealing three separate oligonucleotides, which results in an excess of ssDNA and incomplete annealing of the substrate. These characteristics would likely complicate the interpretation of our results. DNA structure prediction software was utilized to determine that the desired substrate formation correlated with the lowest free energy conformation (32,33). We also performed a FEN1 cleavage assay on the flap substrate to assure proper flap formation, as FEN1 specifically recognizes and cleaves a 5'-flap and does not cleave ssDNA (Supplementary Figure S1). Other substrates used in these studies were designed in a similar manner. The substrates are depicted in Figure 1A.

### Dna2 binding is lower on ssDNA compared to a 5'-flap substrate

To determine whether Dna2 is a non-specific ssDNA endonuclease or has preference for certain structures, Dna2 binding and cleavage assays were performed. Since Dna2 is proposed to play a vital role in Okazaki fragment processing, we first compared Dna2 binding and cleavage on a 5'-flap substrate to that of ssDNA. Cleavage assays were performed with wild-type Dna2 and a nuclease defective Dna2 mutant, E675A (11), was used for the binding assays to prevent cleavage of the flap while still allowing helicase function. As previously observed, Dna2 cleavage activity was similar on the 5'-flap and the ssDNA substrates (Figure 1B and C). However, when



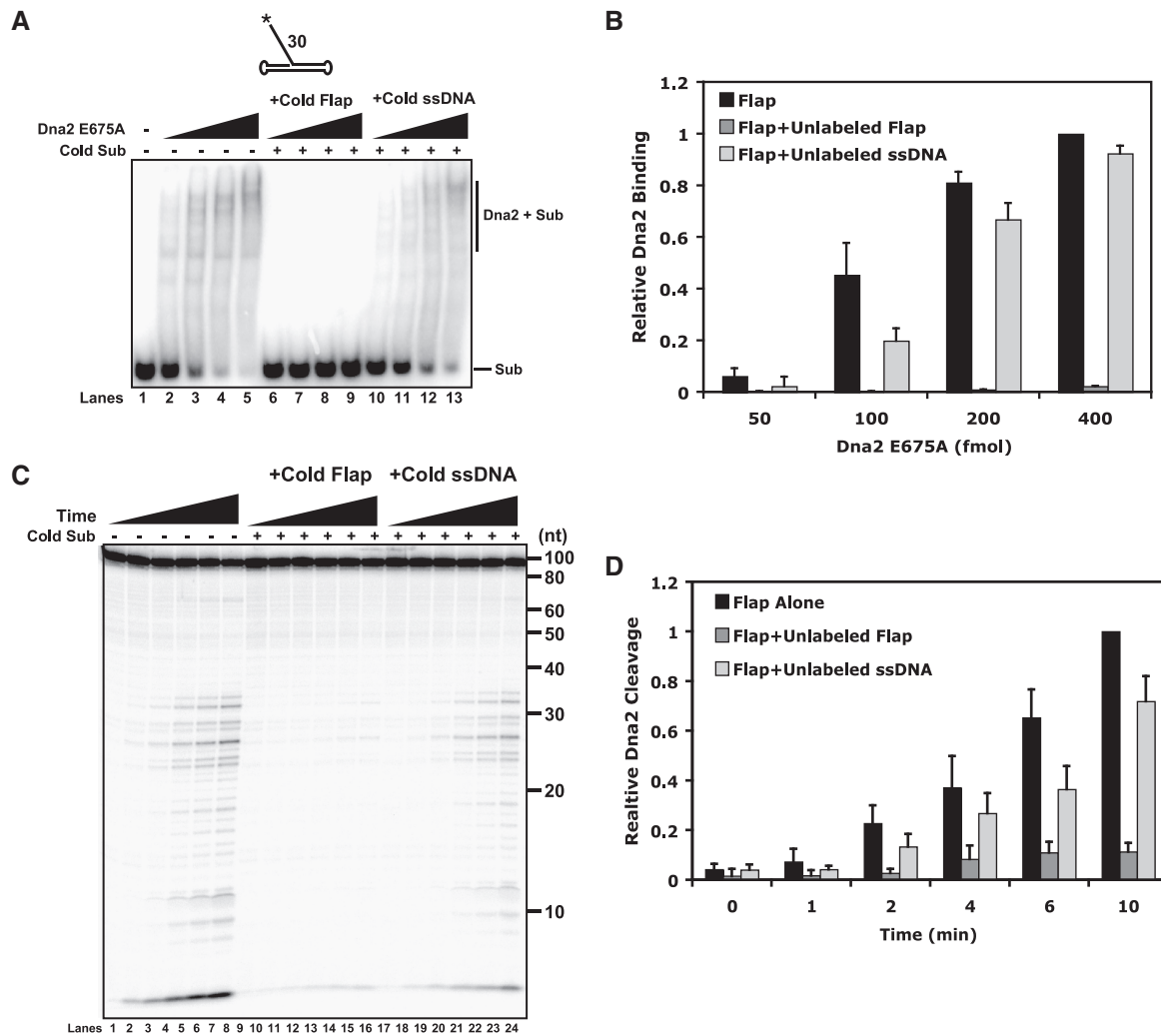
**Figure 1.** Comparison of Dna2 cleavage and binding on a 5'-flap and ssDNA. Reactions contain 5 fmol of the labelled substrate. (A) Depiction of DNA substrates used in the study. (B) Dna2 (25, 50, 100, 200 fmol) cleavage was measured on a 30-nt flap substrate (lanes 1–5) and a 30-nt ssDNA segment (lanes 6–10) by denaturing PAGE. (C) Graphical analysis of (B). Black bars represent cleavage of the 30-nt flap substrate and grey bars represent cleavage of the ssDNA segment. (D) Dna2 E675A (50, 100, 200, 400 fmol) binding activity was measured on a 30-nt flap substrate (lanes 1–4) and a 30-nt ssDNA segment (lanes 6–10) by non-denaturing PAGE. (E) Graphical analysis of (D). Black bars represent cleavage of the 30-nt flap substrate and grey bars represent cleavage of the ssDNA segment. Graphs are an average of at least three experiments and error bars indicate  $\pm$ SD.

we measured DNA binding activity, we observed a significant decrease in the amount of Dna2 bound to the ssDNA compared to the flap (Figure 1D and E). We then measured the relative dissociation constants of Dna2 on the ssDNA versus the flap substrate and observed that Dna2 bound on the ssDNA with an  $\sim$ 3.5-fold lower binding affinity than on the 5'-flap substrate (Table 2).

### Dna2 prefers a flap substrate to ssDNA

We next compared the ability of ssDNA to directly compete with the flap substrate for binding and cleavage by Dna2 (Figure 2). In this assay, the labelled substrate was mixed with a 200-fold excess of unlabelled substrate prior to the addition of Dna2 into the reaction. If Dna2 had no substrate preference then it would equally bind or cleave both the labelled flap and unlabelled ssDNA. This would result in a large reduction in the amount of binding or cleavage of the labelled flap. If the unlabelled ssDNA bound Dna2 with low affinity compared to the labelled flap substrate, then binding or cleavage of the labelled flap substrate would remain similar to that of the labelled substrate in the absence of unlabelled ssDNA.

As shown in Figure 2A, a 200-fold excess of flap substrate competed efficiently for binding to the labelled substrate, even when the level of Dna2 was sufficient to bind nearly all of the labelled substrate in the absence of competitor (Figure 2A, compare lanes 2–5 and 6–9). In contrast, we observed that Dna2 binding to the labelled substrate, in the presence of unlabelled ssDNA, was  $\sim$ 90% of that measured without a competing substrate, at the highest concentration of Dna2 used (Figure 2B, 400 fmol). These findings are striking considering that Dna2 bound ssDNA alone with only a 3.5-fold lower binding affinity compared to the flap (Table 2). From measured affinities, one would expect a substantial reduction in binding to the labelled substrate, since the large excess of unlabelled substrate should overwhelm the difference in binding affinity. Instead, we observed almost complete binding of the labelled flap even in a 200-fold excess of unlabelled ssDNA, suggesting that the Dna2 binding affinity must be at least two orders of magnitude lower on the ssDNA. Determination of binding affinity by gel retardation is a well-respected method, but it is inherently non-equilibrium. Competition assays, while allowing a relative rather than absolute estimation



**Figure 2.** Competition assays between the labelled flap and a 200-fold excess of unlabelled ssDNA. The labelled and unlabelled substrates were mixed prior to the addition of Dna2. Reactions contained 5 fmol of the labelled flap substrate and 1 pmol of the unlabelled substrate. (A and B) Dna2 E675A (50, 100, 200, 400 fmol) binding of the labelled 30-nt flap was measured in either the absence of unlabelled substrate (black bars) or the presence of the unlabelled flap (dark grey bars) or ssDNA (light grey bars). (C and D) Dna2 (50 fmol) cleavage of the labelled 30-nt flap was measured over time in either the absence of unlabelled substrate (black bars) or the presence of unlabelled flap (dark grey bars) or ssDNA (light grey bars). Graphs are an average of at least three experiments and error bars indicate  $\pm$ SD. The labelled substrate is depicted above the gel in (A), with the asterisk indicating the site of the  $^{32}$ P label.

of affinity, make this comparison in equilibrium, and, we feel, provide a more relevant view of the likely distribution of Dna2 among the competing substrates within the cell (35). There, Dna2 is presented with a variety of DNA structures, but presumably has evolved to interact with only the appropriate subset.

We next tested Dna2 cleavage of the flap substrate in the presence of an excess of unlabelled ssDNA (Figure 2C and D). Again, the labelled and unlabelled substrates were mixed prior to the addition of Dna2. Cleavage by Dna2, in the presence of excess unlabelled flap, was significantly diminished to around 10% of that with the labelled flap alone, whereas cleavage of the labelled flap in the presence of excess unlabelled ssDNA was  $\sim$ 75% that of the labelled flap alone (Figure 2D, 10 min). Based on binding results Dna2 cleavage of the labelled flap in the presence of excess unlabelled flap (Figure 2D, gray bars), was expected

to drop to 0.5–1% relative to the labelled flap alone (Figure 2D, black bars). The somewhat greater expected cleavage in the presence of the excess unlabelled flap substrate may have been resulted from the increase in substrate concentration when the competitor was present, accelerating the overall cleavage reaction. Dna2 binding and cleavage were also tested with the labelled ssDNA in an excess of unlabelled flap substrate, which resulted in a reduction of binding and cleavage similar to that of the labelled ssDNA with an excess of unlabelled ssDNA (data not shown). Impressively, these findings demonstrate that Dna2 strongly prefers the 5'-flap substrate to ssDNA.

#### Defining the substrate preference of Dna2

Since Dna2 showed binding and cleavage preference for the 5'-flap over a single-stranded substrate, we attempted

to determine the specific structural features that attract Dna2 to the flap substrate. Substrates were designed which lacked the annealed region upstream of the flap to create a fork, or pseudo-Y, or lacked the entire upstream region to form a 5'-tail (Figure 1A). We also created a double-flap substrate, which has a 1-nt 3'-flap and is the preferred substrate of FEN1 (20). When Dna2 binding and cleavage were tested on these labelled substrates (Table 2 and Figure 3A), we did not observe any significant differences in either binding affinity or cleavage activity.

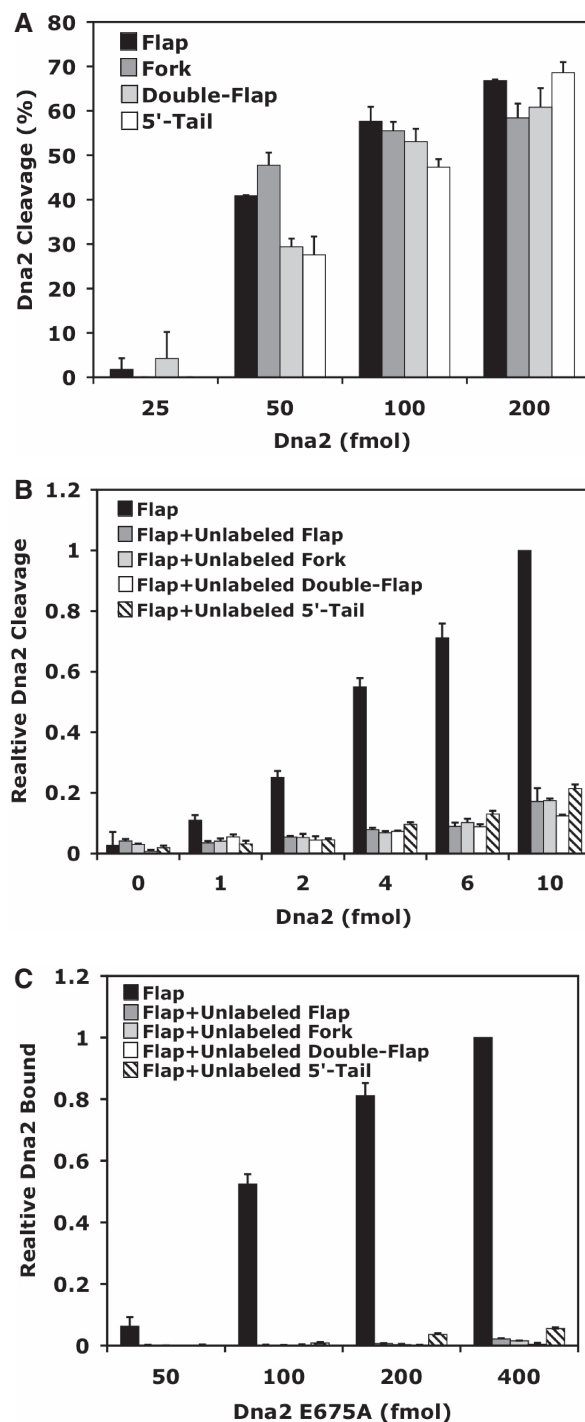
Dna2 binding was also tested on a labelled nick and 20-nt gap substrate (Figure 1A). Cleavage of these substrates was minimal, presumably because these substrates lack a free 5' ssDNA tail for Dna2 entry and cleavage (30). Analysis of these substrates revealed that the dissociation constant was  $\sim 6.5$ -fold higher on the nick than the flap substrate, while the 20-nt gap showed binding comparable to that of the flap (Table 2).

Since the competition approach had previously highlighted differences in substrate preference by Dna2, binding and cleavage of the labelled flap was measured in competition with a 200-fold excess of unlabelled fork, double-flap, or 5'-tail substrates (Figure 3B and C). Interestingly, we observed no significant preference for binding by or cleavage of the flap over the fork or double-flap substrate. However, we did observe an  $\sim 2$ -fold difference in Dna2 binding of the labelled flap in the presence of excess 5'-tail substrate compared to that of excess unlabelled flap.

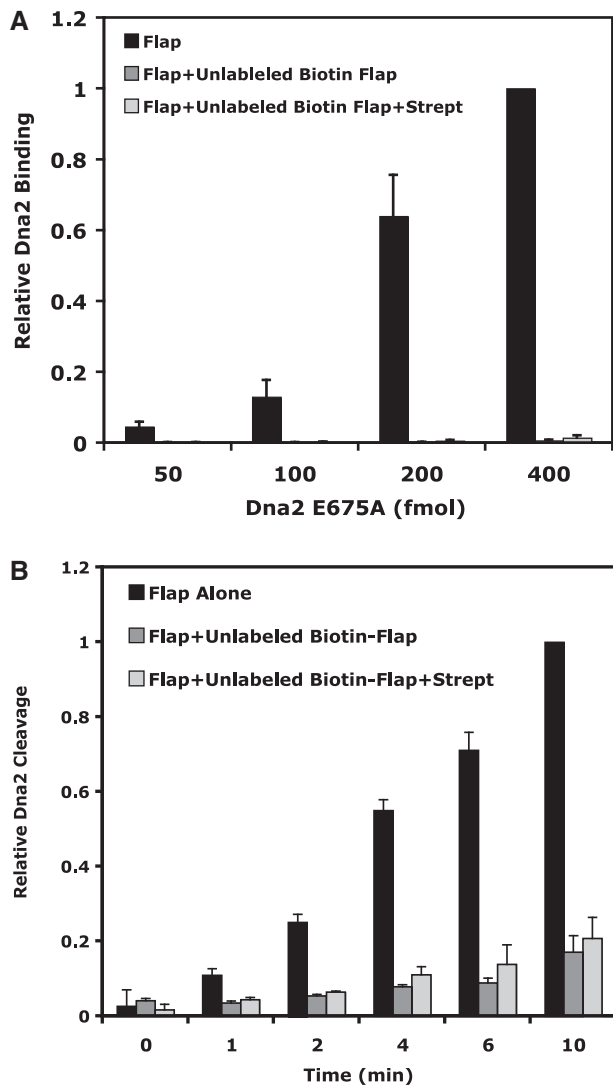
We next considered whether differences in substrate preference would be revealed if the label were shifted between the competing substrates. A labelled fork substrate or labelled double-flap substrate was incubated with an excess of unlabelled flap substrate. We observed no additional preference for either the unlabelled fork or double-flap over the unlabelled flap (Supplementary Figure S2). In summary, Dna2 showed the strongest preference for the 5'-flap and fork substrates, with no additional preference for the double-flap. These results suggest that the key features of Dna2 substrate recognition are located at or near the single-stranded/double-stranded junction of the 5'-flap, i.e. the flap base.

### Recognition of the flap base by Dna2

Next, we tested whether a substrate blocked at the 5'-end, so as to prevent tracking and cleavage on the flap, would still compete with an unblocked flap for binding and cleavage (Figure 4). Previously, we demonstrated that Dna2 still binds to flap substrates even when the 5'-end of the flap is blocked and cleavage cannot occur (23). If the flap base is the primary region of binding specificity, then substrates blocked at the 5'-end should still compete with an unblocked flap substrate for binding. To test our hypothesis, we incubated Dna2 with a mixture of an unblocked labelled flap and an excess of unlabelled flap, which was blocked at the 5'-end with a biotin-streptavidin conjugate. Biotin-streptavidin conjugation was verified by gel-shift analysis (Supplementary Figure S3). We then assayed binding and cleavage of the labelled flap



**Figure 3.** Determination of the structural features required for optimal Dna2 binding and cleavage. (A) Dna2 cleavage analysis of a flap (black bars), fork (dark grey bars), double-flap (light grey bars) and 5'-tail (white bars) substrates. In (B) and (C), the labelled and unlabelled substrates were mixed prior to the addition of Dna2. Reactions contained 5 fmol of the labelled flap substrate and 1 pmol of the unlabelled substrate. (B) Dna2 (50 fmol) cleavage of the labelled 30-nt flap was measured over time in either the absence of unlabelled substrate (black bars) or the presence of unlabelled flap (dark grey bars), unlabelled fork (light grey bars), unlabelled double flap (white bars), or unlabelled 5'-tail (striped bars). (C) Dna2 E675A binding of the labelled 30-nt flap was measured in either the absence of unlabelled substrate (black bars) or the presence of unlabelled substrates as in (B). Graphs are an average of at least three experiments and error bars indicate  $\pm$ SD.



**Figure 4.** Competition assays between the labelled flap and an excess of unlabelled flap blocked at the 5'-end. The labelled and unlabelled substrates were mixed prior to the addition of Dna2. Reactions contained 5 fmol of the labelled flap substrate and 1 pmol of the unlabelled substrates. The biotin-flap was incubated with 5-fold excess streptavidin prior to reaction conditions. (A) Dna2 E675A binding of the labelled 30-nt flap was measured in either the absence of unlabelled substrate (black bars) or presence of the unlabelled biotin-flap (dark gray bars) or the presence of the unlabelled biotin-flap conjugated with streptavidin (light gray bars). (B) Dna2 (50 fmol) cleavage of the labelled 30-nt flap was measured over time in either the absence of unlabelled substrate (black bars) or the presence of unlabelled substrate as in (A). Graphs are an average of at least three experiments and error bars indicate  $\pm$ SD.

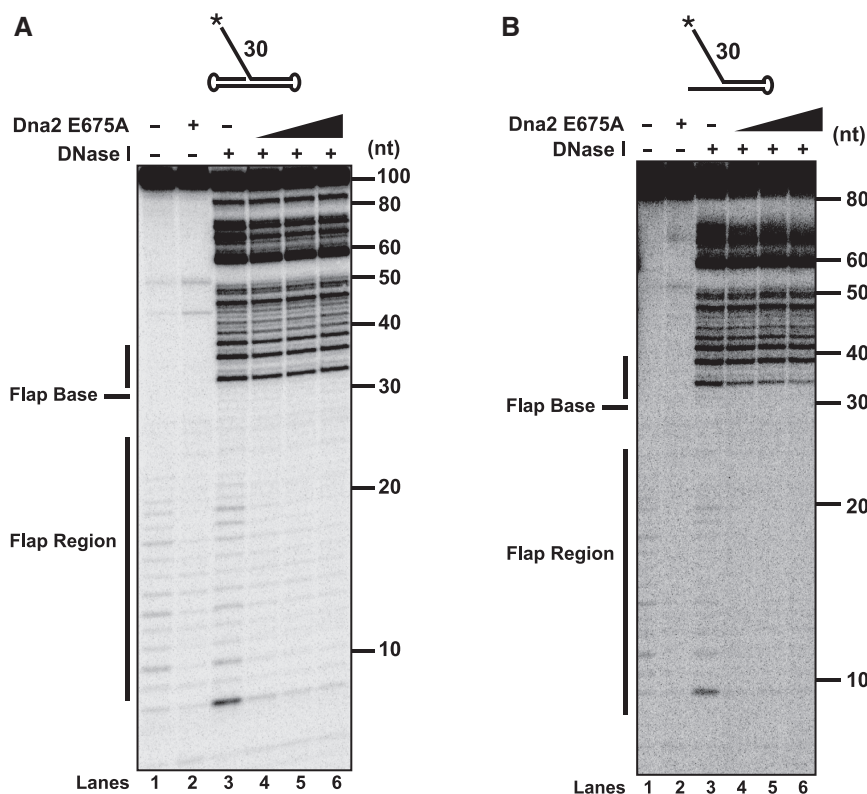
(Figure 4A and B). Both binding and cleavage were reduced to the level of Dna2 binding and cleavage when the excess unlabelled 5'-end blocked flap was mixed with unblocked labelled flap substrate. These results demonstrate that Dna2 has no significant preferential affinity for an unblocked flap over the one blocked at the 5'-end. Taken together with the previous data (Figure 3), these findings point to the single-stranded/double-stranded junction as the primary site of Dna2 recognition and binding.

### Dna2 binds both the flap and downstream-annealed region at the flap base

Finally, we sought to establish the precise regions of Dna2 occupation on the 5'-flap (Figure 5A) or fork (Figure 5B) substrate. Dna2 E675A, lacking nuclease activity, was incubated with the substrate followed by DNase I footprinting analysis. DNase I footprinting analysis involves the addition of limiting amounts of the DNase I enzyme to allow approximately one cleavage reaction per substrate. From the distribution of these cleavages, we obtained the cleavage pattern of DNase I along the substrate (Figure 5, lane 3). This distribution was then compared to that of the substrate bound by Dna2 (Figure 5, lanes 4–6). The decrease in DNase I cleavage products on Dna2 E675A bound substrate compared to the substrate alone represents the footprint, or location, of Dna2 binding on the substrate. After DNase I cleavage, the reactions were then resolved by denaturing PAGE. Comparing the substrate alone with increasing amounts of Dna2, we observed a significant decrease in cleavage products along the flap region. We also observed that the footprint of Dna2 extended into the double-stranded annealed region of the downstream primer (Figure 5). These findings are consistent with DNase I footprinting of the 5'-tail substrate (Supplementary Figure S4A). In addition, we observed a significant inhibition of DNase I cleavage on the entire ssDNA segment and a reduction in cleavage on the nick substrate near the 5'-end of the downstream-annealed region (Supplementary Figure S4B and C). Incidentally, there was also protection of the 3'-end of the fork substrate (Figure 5B, between 60 and 80 nt), consistent with previously reported 3'-end cleavage activity of Dna2 (10,13). Overall, these results suggest that Dna2 interacts with both the single- and double-stranded regions surrounding the flap base.

## DISCUSSION

Dna2 has proposed roles in DNA replication, DNA repair and telomere maintenance. In each of these pathways the nuclease activity of Dna2 is believed to cleave DNA intermediates with very specific structures. An example is Okazaki fragment processing, in which Dna2 aids in the conversion of flap intermediates to nick structures in preparation for ligation (21,25). Dna2 is proposed to work together with FEN1. Early analyses of the properties of FEN1 quickly revealed that it is a structure-specific nuclease (18). It recognizes the 5'-ends of flaps, tracks to the flap base and then binds there prior to cleavage. Dna2 employs a similar tracking mechanism but its terminal cleavage product is 4–6 nt short of the flap base (21,22). It exhibits similar binding and cleavage behavior on ssDNA. The outcome on ssDNA is a cleavage pattern similar to that expected of a random endonuclease. We wondered why one of these specialized nucleases displayed unique binding specificity for its substrates, while the other did not. We report here that appropriately designed competition experiments revealed that Dna2 displays a strong binding and cleavage specificity for 5'-flap, fork and 5' single-stranded extension substrates.



**Figure 5.** Footprinting analysis of Dna2 on the 30-nt flap (A) or fork (B) substrate. Dna2 E675A (200, 400, 800 fmol) was incubated with the flap substrate followed by DNase I footprinting analysis. Reactions were then analyzed by denaturing PAGE. Lanes 1 and 2 are the substrate alone and substrate with Dna2 E675A, respectively. Lane 3 is the flap substrate alone treated with DNase I. FEN1 cleavage was used to identify the flap base.

Our results demonstrate that Dna2 has a substrate specificity as unique as that of FEN1. In fact, the specificities are very similar, in that both Dna2 and FEN1 require a free 5'-end for cleavage and both appear to recognize certain structural features at the flap base, independent of sequence (11,13,19–25).

Moreover, these properties are consistent with the role of Dna2 in DNA repair. Dna2 was recently discovered as a major nuclease required for resection of the 5'-end during DSB repair. The helicase Sgs1 and Dna2 are proposed to work together to create a 3'-overhang, after initial processing by the MRX complex (7,27). In this scenario, Sgs1 would use its helicase activity to separate the DNA strands, resulting in a fork, or pseudo-Y structure. Based on our findings, this is a prime substrate for Dna2 nuclease activity. Dna2 would then cleave the 5'-end of the fork, creating a 3' ssDNA overhang, which is then used for Rad51-dependent homologous recombination.

Both Dna2 and FEN1 are also proposed to be involved in mitochondrial LP-BER repair (6,36). FEN1 had previously been implicated in nuclear LP-BER, where it is proposed to cleave 5'-flap intermediates that arise as a result of strand displacement by either DNA polymerase  $\delta$  or  $\beta$  at the site of DNA damage (37). In the mitochondria, it likely plays a similar role with additional support from Dna2, similar to their roles in DNA replication (38,39). Furthermore, while the exact role of Dna2 at telomeres remains unclear it likely involves the recognition of a 5'-flap or fork intermediate (40,41).

The expected roles of Dna2 in these DNA processing pathways are consistent with its ability to cleave either a 5'-flap or fork, providing it a greater versatility of substrate recognition than that of FEN1 (Figure 3). Nevertheless, these intermediates contain common structural features, particularly that of the single-stranded/double-stranded junction that we find to be the key to Dna2 structure-specific substrate recognition.

While high-resolution structural studies have yet to be performed on Dna2, our results suggest a similar mechanism of DNA substrate recognition and binding to that of FEN1. Dna2, like FEN1, appears to bind at the flap base and likely utilizes this structure to form high-affinity binding contacts. Crystallographic and site-directed mutagenesis studies have revealed that FEN1 uses several specific DNA contacts to coordinate recognition and binding of a 5'-flap substrate (42–50). First, FEN1 contains two dsDNA-binding domains. One binds upstream and the other binds downstream of the 5'-flap. Binding is further stabilized by a 1-nt 3'-flap, which is an element of the preferred substrate of FEN1. After binding at the flap base, the 5'-end of the flap is recognized and bound by a flexible loop within FEN1 (45). This binding induces a conformational shift in the loop region, which encloses the flap and allows tracking (18). Upon reaching the flap base, the active site is stabilized, resulting in cleavage of the 5'-flap to create a nicked DNA substrate.

Several models have emerged concerning the recognition and binding by FEN1 to its DNA substrate.



One proposes that FEN1 initially encounters the 5'-end of the flap followed by tracking toward the flap base (31). FEN1 then binds the base in a way that allows cleavage. Another model proposes that the flap base is the initial site of FEN1 binding (51). Then, upon recognition of the free 5'-end, the flap is threaded through the flexible loop followed by cleavage. We believe that recent data, demonstrating that blockage of the flap at the 5'-end does not abrogate FEN1 binding, is consistent with the latter model (24,52). In addition, our studies here show that Dna2, another tracking enzyme, appears to recognize its substrate prior to tracking and cleavage.

While Dna2 and FEN1 share many important features required for substrate recognition, they also exhibit several pronounced differences. First, Dna2 can bind and cleave ssDNA in isolation. Second, Dna2 cleaves the DNA multiple times along a flap substrate. Third, Dna2 does not require an upstream primer for optimal binding and cleavage. In contrast, FEN1 does not bind a linear ssDNA segment, cleaves at the flap base, and shows significant reduction in cleavage on substrates lacking an upstream primer (18).

Based on the available data, we propose the following model of Dna2 binding and cleavage. We envision that two separate DNA-binding domains facilitate initial Dna2 recognition and binding at the 5'-flap base. One is specific for the double-stranded region downstream of the flap and a second evolved to interact with the ssDNA flap itself. This concept is based on the observations that, while Dna2 bound best to a flap, it still had significant binding affinity for the both a linear unstructured ssDNA segment and a nicked substrate, where binding would involve double stranded DNA (Table 2). These findings suggest that simultaneous binding of both double-stranded and single-stranded regions results in a high affinity binding interaction, which stabilizes Dna2 binding at the single-stranded/double-stranded junction. In addition, DNA footprinting analysis supports this view of Dna2 binding to both the double-stranded and single-stranded regions at the flap base (Figure 5) (23). Furthermore, Dna2 binding is likely stabilized by a kinking or bending of the DNA, similar to FEN1 (43). Dna2 binding would differ slightly from that of FEN1, which requires the dsDNA region upstream of the flap base for optimal binding and cleavage. Since Dna2 does not require the upstream-annealed region, it may have had to evolve more high-affinity interactions with the 5'-flap than those employed by FEN1 (Figure 3B). Dna2 binding at these sites may have prevented the active site from evolving an orientation that could completely remove the 5'-flap. Alternatively, the location of the active site for cleavage with respect to the binding sites on Dna2 may simply have evolved differently from that of FEN1, precluding cleavage of the entire 5'-flap.

After binding the flap base, Dna2 must then recognize the 5'-end of the flap, likely through a third DNA-binding domain near the active site of the nuclease domain. The 5'-end region is then threaded, or looped, through the active site with periodic cleavage events. The flap then reaches a terminal length at which point Dna2 cannot cleave. In the case of Okazaki flap processing, FEN1

would then dissociate the Dna2 from the flap, and cleave at the base of the short flap to create a nick for ligation (23,24). During DNA repair and telomere maintenance, other proteins may play a similar role as that of FEN1 in removing Dna2 from the flap for final processing events.

In summary, Dna2 acts in DNA processing pathways that conserve genome stability. Here, we have demonstrated that Dna2 preferentially binds to 5'-flap or fork substrates, with specificity exactly consistent with its expected cellular roles. Based on our findings, we propose a model of Dna2 substrate recognition and binding. While additional studies are required to verify this model, including the identification of DNA-binding domains and high-resolution structural studies of Dna2, our findings reveal the unique substrate specificity of Dna2.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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