Coupling of DNA Helicase and Endonuclease Activities of Yeast Dna2 Facilitates Okazaki Fragment Processing

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Saccharomyces cerevisiae Dna2 possesses both helicase and endonuclease activities. Its endonuclease activity is essential and well suited to remove RNA-DNA primers of Okazaki fragments. In contrast, its helicase activity, although required for optimal growth, is not essential when the rate of cell growth is reduced. These findings suggest that DNA unwinding activity of Dna2 plays an auxiliary role in Okazaki fragment processing. To address this issue, we examined whether the Dna2 helicase activity influenced its intrinsic endonuclease activity using two mutant proteins, Dna2D657A and Dna2K1080E, which contain only helicase or endonuclease activity, respectively. Experiments performed with a mixture of Dna2D657A and Dna2K1080E enzymes revealed that cleavage of a single-stranded DNA by endonuclease activity of Dna2 occurs while the enzyme translocates along the substrate. In addition, DNA unwinding activity efficiently removed the secondary structure formed in the flap structure, which was further aided by replication protein A. Our results suggest that the Dna2 unwinding activity plays a role in facilitating the removal of the flap DNA by its intrinsic endonuclease activity.

Okazaki fragment synthesis requires the action of DNA polymerase α-primase, δ, and/or ε with proliferating nuclear antigen and replication factor C (1–3). Polymerase α, tightly complexed with DNA primase, plays a role in the initiation of DNA synthesis by providing RNA-DNA primers for both leading and lagging strands. Okazaki fragments, after removal of primer RNAs, are ligated together through a process called Okazaki fragment maturation. This process requires the combined action of Fen-1, RNase HI, and DNA ligase I (3–8). Genetic studies revealed that Dna2 of Schizosaccharomyces pombe is likely to be involved in Okazaki fragment maturation by virtue of its genetic and physical association with several enzymes involved in Okazaki fragment maturation (9–11). The essential DNA2 gene of S. cerevisiae encodes a 172-kDa protein with characteristic DNA helicase motifs, and the Dna2 enzyme possesses DNA helicase activity (12). Cells harboring temperature-sensitive alleles of DNA2 arrested at either G2/M with a duplicated DNA content at the restrictive temperature (9, 13). The recombinant S. cerevisiae Dna2 protein purified from insect cells intrinsically contained strong single-stranded DNA specific endonuclease activity in addition to helicase activity (14).

Further characterization of the endonuclease activity of Dna2 revealed that Dna2 is not a structure-specific endonuclease, unlike Rad27, a yeast homolog of mammalian Fen1, but prefers to cleave single-stranded DNA (ssDNA) with free ends (15). Importantly, the endonuclease activity of Dna2 is stimulated by the presence of a terminal RNA segment at the 5′ end of ssDNA (15). Moreover, cleavage of the 5′ end of Okazaki fragments by Dna2 was readily observed in conjunction with DNA polymerases capable of displacement DNA synthesis. This unique property of Dna2 appears to ensure the complete removal of the initiator RNA segment on Okazaki fragments, providing a biochemical role of Dna2 endonuclease activity in Okazaki fragment processing (15). Recently, we discovered that replication protein A (RPA) suppressed the temperature-sensitive phenotype of the mutant dna2Δ405N allele that lacked the N-terminal 405-amino acid region of DNA2 (16, 17). Subsequently we showed that RPA promotes the endonuclease, switching between Dna2 and Fen1 in the following manner (17). RPA initially binds to the 5′ end region containing RNA primers displaced by polymerase δ and then recruits Dna2, resulting in increase in Dna2-catalyzed cleavage of the primer RNAs. The cleavage reaction causes RPA to dissociate from the shortened flap, permitting Fen1 to access the remaining flap (17). These findings suggested a new model for Okazaki fragment processing in which the endonuclease activity of Dna2, in collaboration with Fen1, plays a critical role in Okazaki fragment maturation (17). In support of this critical role of the endonuclease activity of Dna2, the endonuclease-deficient Dna2 did not support cell growth (18, 19).

In contrast to the important role of the Dna2 endonuclease activity in Okazaki fragment processing, the properties of the Dna2 helicase activity are not well characterized. A point mutation in the ATP binding motif (K1080E) of DNA2 led to inactivation of its ATPase and helicase activities and rendered mutant cells not viable (14, 20), indicating the critical role of the unwinding activity in vivo. However, mutant cells expressing Dna2K1080T were capable of growth in media when lactate and glycerol were used as the carbon source instead of glucose,

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The abbreviations used are: ssDNA, single-stranded DNA; ssDNA, single-stranded circular DNA; RPA, replication protein-A; nt, nucleotide(s); SSB, single-stranded DNA-binding protein.
suggesting that Dna2 appears to act in lagging strand metabolism in a role that is optimal with, but does not require, full helicase activity (21). Thus, the helicase activity of Dna2 may play a supportive role that becomes essential in rapidly growing cells.

In this paper, we have characterized the biochemical properties of the Dna2 helicase activity in an attempt to understand the role of its DNA unwinding activity during Okazaki fragment processing. We present evidence that the helicase activity of Dna2 facilitates its intrinsic endonuclease activity by resolving secondary structures present in the primer-RNA-containing flap during Okazaki fragment maturation.

**EXPERIMENTAL PROCEDURES**

_Oligonucleotides, DNA, and Nucleoside Triphosphates—_All oligonucleotides used for the construction of various DNA and RNA substrates were synthesized commercially and gel-purified before use. The sequences of all oligonucleotides used in this study are listed in Table I. Oligonucleotides used to prepare substrates, the positions of radioisotope labels in the substrates, and the substrate structures were as described (14). A mutant Dna2D657A protein devoid of endonuclease activity was prepared as described previously (18). RPA was purified from a protease-deficient yeast strain BJ2168 (MATa, ura3-52, trpl-1,363, leu2-Δ, prb1-1222, pep4-3, plc1-407, gal2) as described (22).

_Preparation of DNA Helicase and Nuclease Substrates—_The DNA substrates used to examine the DNA unwinding activity of Dna2 protein were constructed by hybridizing the oligonucleotides listed in Table I to X174 sscDNA as described (23). The various partial duplex substrates used to characterize the helicase and endonuclease activities of Dna2 were prepared as described previously (14) using the synthetic oligonucleotides listed in Table I. Labeling of the 5′- or 3′-ends of the oligonucleotides in the substrates was as described (14).

_ATPase, Helicase, and Endonuclease Assays—_ATP hydrolysis was measured in reaction mixtures (20 μl) that contained 50 mM Tris-Cl (pH 7.8), 0.3 mM MgCl2, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 250 μM cold ATP, 20 nM [γ-32P]ATP, and the indicated amounts of cofactor DNAs. After incubation with 2 ng (10 fmol) of Dna2 at 37 °C for 10 min, aliquots (2 μl) were spotted onto a polyethyleneimine-cellulose plate (J. T. Baker Inc.), which was then developed in a TBE (45 mM Tris-base, 89 mM boric acid, 2 mM EDTA) buffer. The products were subjected to electrophoresis for 1.5 h at 150 V through 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (45 mM Tris-base, 89 mM boric acid, 2 mM EDTA) buffer. The DNA products were analyzed using an Autoradiograph (Molecular Dynamics).

Helicase assays were performed with the endonuclease-deficient mutant enzyme, Dna2D657A, unless otherwise indicated. The standard reaction mixture (40 μl) contained 50 mM Tris-Cl (pH 7.8), 2 mM MgCl2, 2 mM dithiothreitol, 0.25 μg/ml bovine serum albumin, 20 μM ATP, and 15 fmol of helicase substrate. When necessary, the concentrations of ATP and/or MgCl2 were varied as indicated in the individual experiments. The reactions were preincubated at 37 °C for 5 min and initiated by the addition of enzyme. Reactions were stopped with 6× stop solution (8 μl; 60 mM EDTA (pH 8.0), 40% (w/v) sucrose, 0.6% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanol). The reaction products were subjected to electrophoresis for 1.5 h at 150 V through 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (45 mM Tris-base, 89 mM boric acid, 2 mM EDTA) buffer. The DNA products were analyzed using an Autoradiograph (Molecular Dynamics).
The synthetic double-stranded DNA (poly(dI-dC)) the least effective. Both wild type and mutant Dna2 had a slight decrease (6%) in ATPase activity as compared with wild type enzyme. This difference, however, appears insignificant and within an error range of measurement since several other mutations that affected the endonuclease activity did not affect ATPase activity noticeably (18). In the presence of supercoiled DNA by both enzymes (data not shown). These results suggest that the D657A mutation did not alter the ATPase activity of Dna2 nor, most likely, the helicase activity.

**Results**

**Characterization of ATPase Activity of Endonuclease-deficient Dna2**—Because the potent endonuclease activity associated with Dna2 interfered with the measurement of its helicase activity, all of the experiments described below are carried out with the mutant Dna2D657A, an enzyme devoid of detectable endonuclease activity (18). To ensure that the point mutation in Dna2D657A did not alter its ability to hydrolyze ATP, we compared the requirements for ATP hydrolysis by the mutant Dna2D657A and wild type Dna2 enzymes (Table II). The mutant Dna2 had a slight decrease (~6%) in ATPase activity as compared with wild type enzyme. This difference, however, appears insignificant and within an error range of measurement since several other mutations that affected the endonuclease activity did not affect ATPase activity noticeably (18). Both wild type and mutant enzymes were inhibited similarly by increasing the concentrations of NaCl; both enzymes were dependent on Mg$^{2+}$, whereas Mn$^{2+}$ was less effective than Mg$^{2+}$, and Ca$^{2+}$ was inactive. The DNA effectors that supported ATP hydrolysis included M13 ssDNA, poly(dT), poly(dA), and poly(dG) with M13 ssDNA the most effective and poly(dG) the least effective. Both wild type and mutant enzymes did not hydrolyze ATP in the presence of synthetic single-stranded RNAs such as poly(U), poly(A), and poly(G). The synthetic double-stranded DNA ((poly(dG-C)) supported ATP hydrolysis similarly (44–56%, wild type; 48–59%, Dna2D657A). However, this activity is most likely due to the presence of free ssDNA regions that did not form a duplex region (see below). Supercoiled double-stranded plasmid DNA supported ATP hydrolysis but with a lower efficiency than M13 ssDNA. Linearized plasmid DNA did not support ATP hydrolysis with either enzymes (Table II). Therefore, we conclude that Dna2 requires single-stranded DNA for the hydrolysis of ATP.

**Directionality of Dna2 Unwinding**—To optimize the unwinding reaction catalyzed by Dna2D657A, we determined the ratio of Mg$^{2+}$ to ATP. The helicase activity of Dna2D657A was measured at various concentrations of ATP and Mg$^{2+}$ (Fig. 1A). Maximal unwinding activity was observed when the concentrations of Mg$^{2+}$ were equimolar to those of ATP (Fig. 1A). The optimal concentrations of Mg$^{2+}$ increased in proportion to increasing concentrations of ATP (Fig. 1A). Based upon these results, all subsequent unwinding reactions were carried out at 2 mM of ATP and 2 mM MgCl$_2$.

We have shown previously that wild type Dna2 translocated in the 5’ to 3’ direction using reaction conditions in which the nuclease activity was suppressed substantially by lowering the ratio of ATP/MgCl$_2$ (15). We reexamined the polarity of Dna2 helicase activity under the above optimized helicase condition (2 mM ATP and 2 mM MgCl$_2$) using Dna2D657A and oligonucleotide substrates containing either 5’- or 3’-ssDNA tail (Fig. 1B). Consistent with our previous results (15), Dna2D657A unwound the substrate with a 5’-ssDNA tail only, indicating that Dna2 translocates in the 5’ to 3’ direction (Fig. 1B).

**Influence of a Preexisting Fork Structure on Duplex Unwinding**—In previous studies, the influence of a fork structure on the Dna2 helicase activity could not be evaluated due to the presence of endonuclease activity, which preferentially cleaved the ssDNA tail in the forked substrate (15). The availability of the Dna2D657A enzyme containing helicase activity only permitted us to examine this question. For this purpose, a substrate containing a 25-nt 3’-ssDNA tail was prepared. Theoretically Dna2 can interact with this fork structure when it translocates in the 5’ to 3’ direction along the template ssDNA as illustrated in Fig. 2A. The fork structure containing a 3’-ssDNA tail, however, was displaced at the same rate by Dna2D657A as was a flush structure lacking a fork structure (Fig. 2). This result demonstrates that Dna2 is neither dependent on nor stimulated by the fork structure per se. Next we examined the effect of substrates containing 5’-ssDNA tails on the helicase activity of Dna2. For this purpose, several

### Table II

<table>
<thead>
<tr>
<th>Additions or omissions</th>
<th>Amount added</th>
<th>Relative activity</th>
</tr>
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<tbody>
<tr>
<td>Add NaCl</td>
<td>0, 25, 50, 100, 200 mM</td>
<td>100, 82, 78, 41, 4</td>
</tr>
<tr>
<td>Omit MgCl$_2$</td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td>Add EDTA</td>
<td>2 mM</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Add MnCl$_2$</td>
<td>2 mM</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Add CaCl$_2$</td>
<td>2 mM</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Omit M13 ssDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Add poly(dt)</td>
<td>50 ng</td>
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<tr>
<td>Add poly(dA)</td>
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<td>Add poly(dG)</td>
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<td>Add poly(U)</td>
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<td>Add poly(A)</td>
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<tr>
<td>Add dUC19 RF1</td>
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<td></td>
</tr>
<tr>
<td>Add dUC19 RFIII</td>
<td>100, 50 ng</td>
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</tbody>
</table>

* The amount of ATP hydrolyzed by wild type Dna2 with 50 ng of M13 ssDNA (2004 pmol) represented 100%.

45 mM boric acid, 1 mM EDTA). The gel was dried on DEAE-cellulose paper and subjected to autoradiography. Labeled DNA products were quantitated with the use of a PhosphorImager.

The reaction conditions used to examine Dna2 endonuclease activity were the same as those used for the DNA unwinding reaction except that ATP was omitted. Endonuclease assays were carried out at 37 °C for 5 min with either wild type Dna2 or Dna2K1080E, an ATPase/helicase-deficient mutant enzyme. Reactions were stopped by the addition of 2× stop solution (95% formamide, 20 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol). The nucleolytic products were boiled for 1 min and subjected to electrophoresis for 1.5 h at 35 W in 1× TBE through 12 or 20% denaturing polyacrylamide gel containing 7 M urea as described previously (14). To examine the effects of ATP on the endonuclease activity of Dna2, ATP (2 mM) was included as indicated.

In all of the above reactions, Dna2 was diluted to the appropriate concentrations before use with 50 mM Tris- HCl (pH 7.8) containing 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 10% glycerol, 0.5 mM NaCl, and 0.02% Nonidet P-40. Nonidet P-40 stabilizes Dna2 especially at low protein levels.
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Polarization of helicase activity. The helicase activity of Dna2D657A was measured under various conditions. The concentrations of ATP used were 0.5 (open circle), 2 (closed circle), and 5 mM (open square). The experiment was repeated three times, and one representative result is shown. B, polarity of helicase activity. The schematic structures of substrates used are shown at the top of the figure. Oligonucleotides 8 or 11 (see Table I) were annealed to oligonucleotide 12 to construct the 5′-overhang partial duplex DNA substrate, respectively. Both substrates contained 25-nt oligo(dT) overhang partial duplex DNA substrate. The asterisks indicate the position where the labeled oligonucleotides migrated. The amount of substrate unwound is presented at the bottom of the figure.
unwinding efficiency decreased dramatically (15-fold less than that observed with the 30-bp duplex). The addition of *E. coli* SSB inhibited Dna2-catalyzed unwinding (Fig. 4C, lanes 1–5), and the addition of RPA did not affect unwinding efficiency of the 91-bp duplex substrate (Fig. 4C, lanes 6–8). Therefore, the result that longer primers are displaced less efficiently than shorter ones is not due to the rewinding of displaced DNA after the unwinding action of the enzyme.

To examine whether Dna2 acts in a processive manner, we carried out substrate-challenge experiments by adding a 10-fold molar excess of the 4X174 template to the reactions at different time points (0, 2, and 5 min) after initiation of the unwinding reaction. As shown in Fig. 4D, the unwinding activity of Dna2D657A was immediately blocked by the addition of excess template ssDNA at any time point after initiation. This result demonstrates that Dna2 is a weak helicase and unwinds duplex DNA in a highly distributive manner.

**Effect of a Terminal RNA Segment of a ssDNA Tail on Dna2 Helicase Activity—**Because RNA did not support ATPase activity of Dna2 (Table II), whereas a terminal RNA segment on a ssDNA tail stimulated the endonuclease activity (15), we examined whether the 5′-terminal RNA could also stimulate unwinding activity of Dna2 using flap-structured substrates (shown in Fig. 5A). The terminal 12-mer oligo(U) RNA segment in the chimeric flap (total, 25 nt) neither stimulated nor inhibited helicase activity compared with that obtained with a flap of 25-nt DNA (Fig. 5A). This contrasts with the finding that the same terminal RNA segment stimulated the endonuclease ac-

![Diagram A](Image)

**FIG. 3. Effects of 3′ tails on duplex unwinding by Dna2.** A, unwinding of different substrates. 4X174 DNA substrates with a 30-bp duplex region and varying lengths of a 5′ tail (0, 10, 25, or 40 nt) were prepared by annealing either the oligonucleotides 1, 5, 8, or 9 to 4X174 ssDNA, respectively. The *asterisks* indicate 32P-labeled ends. The reactions were performed with 80 ng (450 fmol) of Dna2D657A as described in Fig. 2. B denotes boiled substrate controls. The arrows indicate the positions where the labeled oligonucleotides migrated. The direction of unwinding (arrows) by Dna2 (hatched circle) on the two different substrates is shown at the right of the figure. B, quantitation of the displacement reaction. The amounts of substrate unwound in A are presented. The symbols indicate substrates with flush end (open circle), 10-nt oligo(dT) tail (closed circle), 25-nt oligo(dT) tail (open square), and 40-nt oligo(dT) tail (closed square). C, quantitation of the displacement reaction contributed by the presence of the 5′ tail. The differences between the unwinding of flush and 5-tailed substrates were plotted as a function of incubation time.

Stimulation of Dna2 Helicase Activity by RPA at Physiological Salt Concentrations—To analyze the effect of RPA on the unwinding activity of Dna2, we examined the rate of unwinding in the presence and absence of RPA. The stimulatory effect of RPA was salt-dependent as shown in Fig. 6A. In the presence of low concentrations of NaCl (25 mM), RPA neither stimulated nor inhibited the unwinding activity of Dna2D657A (Fig. 6A, lanes 2–5). In contrast, RPA stimulated unwinding activity of Dna2D657A in the presence of 125 mM NaCl (Fig. 6A, lanes 8–11). The addition of 125 mM NaCl nearly completely inhibited the unwinding activity of Dna2 in the absence of RPA (Fig. 6A, compare lanes 2 and 8), and the addition of RPA efficiently reversed the inhibitory effects of salt (Fig. 6A, lanes 9–11). RPA alone did not unwind the substrate (Fig. 6A, lanes 6 and 12). In a time course analysis, the addition of RPA markedly (10–30-fold) stimulated the formation of ssDNA products in the presence of 125 mM NaCl (Fig. 6B, compare lanes 1–5 to 6–10). In contrast, the addition of *E. coli* SSB inhibited unwinding of duplex even in the presence of high salt (Fig. 6B, lanes 12–16). This result excludes the possibility that RPA is required simply to overcome the effect of high salt that stabilizes duplex DNA. Our result that Dna2 is stimulated by RPA but inhibited by *E. coli* SSB is consistent with our previous observation that RPA stimulates the complex formation of Dna2 with a substrate DNA, whereas *E. coli* SSB interferes the complex formation (17). Therefore, it is most likely that RPA directly stimulates DNA helicase activity by direct protein-protein interaction. RPA also stimulated unwinding of 4X174-based substrates by Dna2 to the similar extent (data not shown).

**Helicase and Endonuclease Activities Are Coupled—**The weak helicase activity of Dna2 with a highly distributive nature may suggest that DNA helicase may act as a motor protein to assist delivery of the Dna2 endonuclease activity near the flap junction during Okazaki fragment processing. This positioning may permit the endonuclease to remove the flap as much of the flap substrate as possible. If this is the case, cleavage and unwinding are most likely to occur in a coupled manner. To address this question, we used a flap-structured DNA substrate in which only one single strand was available for both activities. As shown in Fig. 7A, the size of cleavage products formed by wild type Dna2 in the absence of ATP ranged from 3 to 25 nt (Fig. 7A, lanes 2 and 3). In contrast, the average size of products formed in the presence of ATP were significantly larger than those obtained in the absence of ATP (Fig. 7A, lanes 4 and 5). Some cleavage products are longer than the length of the flap (25 nt). The cleavage pattern generated by Dna2K1080E, a mutant enzyme that lacks helicase activity, was not altered by the addition of ATP except that cleavage was slightly inhibited by the presence of ATP (Fig. 7A, lanes 6 and 7). As expected, the endonuclease-deficient Dna2D657A did not produce any cleaved products (Fig. 7A, lane 8), although significant unwinding of duplex DNA was detected (data not shown). When the two mutant enzymes were added together, they did not yield any larger products that could arise by the translocation of the enzyme toward the flap junction and/or subsequent unwinding of the duplex region.

tivity of Dna2 (Fig. 5B; see also Ref. 15) when the overall length of the 5′ flap remained constant. It is noteworthy that the 25-nt chimeric flap substrate was unwound more efficiently (3-fold) than the 13-nt DNA flap substrate (Fig. 5A), suggesting that the overall length of the 5′ tail is important in acting as an entry site for Dna2. In the presence of lower concentrations (0.1–0.5 mM) of MgCl2, the stimulatory effects of the terminal RNA on the endonuclease activity of Dna2 was more dramatic (15). Under this condition, however, the unwinding activity of Dna2D657A was too weak to be reliably measured (Fig. 1A).
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(Fig. 7A, lanes 9–12). This result demonstrates that cleavage and translocation/unwinding occur in a coupled manner when Dna2 utilizes the same strand for both cleavage and translocation. The coupled reaction appears to be essential in vivo since DNA2-deleted cells were not viable when the two plasmids, one expressing Dna2K1080E and the other Dna2D657A, were present in the same cell (Fig. 7B). Interestingly, when both mutation plasmids were present, cells were not viable even when grown in a lactate/glycerol media, although the helicase-defective mutant cells grew (Fig. 7B). The significance of this result is discussed below.

Previously, we showed that labeled 3′ flush ends of partial duplex DNAs were cleaved by Dna2 only in the presence of ATP (14). The likely scenario involved in this process includes the action of Dna2 helicase activity; the partial melting of 3′ ends of duplex DNAs by the 5′ to 3′ unwinding action of Dna2 generated short ssDNAs that were susceptible to Dna2 endonuclease activity. We found this reaction does not require a concerted action of the helicase and endonuclease activities of Dna2, since a combination of mutant Dna2 proteins, Dna2D647A (helicase−, endonuclease−) and Dna2K1080E (helicase−, endonuclease+), was as efficient as wild type Dna2 (Fig. 8, compare lanes 4–5 and 14–15). Therefore, this in vitro activity is not an essential function of Dna2 since the two mutant alleles, Dna2D647A and Dna2K1080E, do not complement each other in vivo (Fig. 7B).

Resolution of a Secondary Structure Present in the Flap DNA by Dna2—The coupled helicase-endonuclease action of Dna2 suggests that Dna2 helicase activity may play a crucial role by removing secondary structures present in the flap that would normally be resistant to Dna2 endonuclease, thereby assisting Dna2 endonuclease activity. To test this possibility, we prepared a flap substrate containing a pre-formed secondary structure as illustrated in Fig. 9A. The substrate was labeled at the 3′ end of the flap-containing strand, and the secondary structure consisted of 10-bp duplex in the central region of the flap DNA. In the absence of ATP, wild type Dna2 (0.5 or 2.5 fmol) cleaved the terminal ssDNA tail only, resulting in the cleavage products of 50 nt or longer (Fig. 9A, lanes 2 and 3; oligonucleotides 1, 2, or 4 were annealed to ΦX174 sscDNA to construct the 30-, 52-, or 91-bp partial duplex substrates, respectively. The asterisks indicate 32P-labeled ends. Reactions (200 µl) were carried out with 50 ng (270 fmol) of Dna2D657A and 75 fmol of substrates as described in Fig. 2. B denotes boiled substrate controls. The arrow indicates the positions where the labeled oligonucleotides migrated. B, quantitation of the displacement reaction. The extent of substrate unwound in A is shown. The circle, square, and triangle indicate 30-, 52-, and 91-bp partial duplex ΦX174 substrates, respectively. C, different effects of SSB or RPA on the Dna2 helicase activity. Reactions (40 µl) containing 15 fmol of 91-bp partial duplex substrate shown in A were preincubated at 37 °C for 5 min with increasing amounts (400, 800, and 1600 fmol, triangles) of E. coli SSB or yeast RPA. The unwinding reaction was initiated by addition of Dna2D657A (40 ng, 220 fmol) followed by incubation at 37 °C for 10 min. The reactions shown in lanes 5 and 9 are SSB (125 ng, 1600 fmol)- or RPA (200 ng, 1600 fmol)-alone controls, respectively. Products were analyzed on 10% polyacrylamide gel. B and S denote boiled substrate and substrate alone controls, respectively. An arrow indicates the position where the labeled oligonucleotide migrated, and the amounts of substrate unwound are presented at the bottom of the figure. D, substrate-challenge experiments. Reaction mixtures (240-µl) were preincubated at 37 °C for 5 min with 90 fmol of ΦX174 DNA substrate with a 30-bp duplex region and initiated by the addition of Dna2D657A (60 ng, 330 fmol). The competitor DNA (ΦX174 sscDNA, 600 fmol) was added at the indicated time point (arrows). Aliquots (40-µl) were withdrawn at each time point, and the products were analyzed by electrophoresis through a 10% polyacrylamide gel. Closed circles indicate the amounts of substrate unwound in the absence of the competitor DNA. Open circles, closed squares, and open squares indicate the amounts of substrate unwound after the addition of the competitor DNA at 0, 2, and 5 min, respectively. The mean of three independent experiments is shown with error bars.

Fig. 4. Limited unwinding activity of Dna2 helicase. A, influence of duplex length on the displacement reaction. Schematic structures of various substrates used are shown at the top of the figure. The
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Fig. 5. Influence of the presence of a 5′-terminal RNA segment on a flap substrate on the Dna2 enzyme activities. To construct the flap-structured substrates with different 5′ tail length, both downstream (either oligonucleotides 6, 7, or 8) and upstream primers (oligonucleotide 15) were annealed to the template primer (oligonucleotide 14). The terminal RNA segment (12 nt) of the flap is indicated by the wavy line. The asterisks denote 32P-labeled ends. A, influence of forks structures on DNA helicase activity. The standard reaction mixtures (40 μl) containing 2 mM ATP and 15 fmol of substrate were incubated with the indicated amounts of Dna2D657A at 37 °C for 5 min. The products were analyzed on 10% polyacrylamide gel. B, influence of fork structures on the endonuclease activity of wild type Dna2. The endonuclease reaction mixtures (40 μl) containing 15 fmol of substrate were incubated with the indicated amounts of Dna2 at 37 °C for 5 min. The products were analyzed on a high resolution sequencing gel.

Dna2 is a ssDNA-specific endonuclease (14, 15). When we added ATP to the reaction, cleavage occurred within the region containing the secondary structure, generating products shorter than 50 nt (Fig. 9A, lanes 6 and 7; indicated by the lower bracket). In the presence of ATP, the addition of RPA further facilitated removal of the secondary structure by Dna2 (Fig. 9A, lanes 8 and 9). The formation of these products required intact helicase activity, since they were not generated with the mutant Dna2 protein, Dna2K1080E, devoid of helicase (Fig. 9A, lanes 10 and 11). The presence of RPA alone, in the absence of ATP, also allowed Dna2 to cleave the flap past the secondary structure (Fig. 9A, lanes 4–5 and 12–13). This is consonant with the fact that RPA destabilizes duplex DNA and stimulates the endonuclease activity of Dna2 (17). In support of these biochemical data, multicopy expression of the large subunit of RPA (RFA1) suppressed the growth defect due to the mutation of the Dna2 helicase (Fig. 9B). When the plasmid containing wild type DNA2 was counter-selected by 5-fluoroorotic acid, neither Dna2K1080E nor multicopy expression of RFA1 rescued the
lethal phenotype of the DNA2-deleted strain. When both Dna2K1080E and RPA1 were present, cells grew as efficiently as wild type cells (Fig. 9B). These data present strong evidence that the helicase activity of Dna2 collaborates in vivo with RPA to facilitate removal of secondary structure.

**DISCUSSION**

In this report, we have determined a number of biochemical properties of the Dna2 helicase activity that contribute to its in vivo role in Okazaki fragment processing. We analyzed the ability of endonuclease-deficient Dna2D647A to hydrolyze ATP and compared this with wild type Dna2 as summarized in Table II. No difference was noted. Interestingly, both enzyme preparations were stimulated by oligo(dT) as short as 16 nt as efficiently as M13 ssDNA (data not shown). The rate of ATP hydrolyzed was comparable regardless of the size of the oligo(dT) chains, provided the same molar concentrations of each oligo(dT) was used. The fact that short oligonucleotides supported ATP hydrolysis as efficiently as long M13 ssDNA indicates that ATP hydrolysis is most likely to occur when Dna2 interacts with cofactor DNAs with a rapid on and off rate. If ATP is hydrolyzed by processive translocation of Dna2 along template DNA without dissociating from it, then M13 ssDNA would support ATP hydrolysis much more efficiently than short oligonucleotides. Therefore, we conclude that Dna2 hydrolyzes ATP in a distributive fashion, consistent with its unwinding properties (shown in Fig. 4). These findings suggest that the D657A mutation did not affect the DNA-dependent ATPase activity nor the helicase activity of Dna2.

The properties of the helicase activity of Dna2D647A are consonant with the action of the endonuclease of Dna2 during lethal phenotype of the DNA2-deleted strain. When both Dna2K1080E and RPA1 were present, cells grew as efficiently as wild type cells (Fig. 9B). These data present strong evidence that the helicase activity of Dna2 collaborates in vivo with RPA to facilitate removal of secondary structure.

**FIG. 7.** The influence of the action of helicase on endonuclease of Dna2. A, the flap substrate used is the same as that described in Fig. 5. The substrate (15 fmol) was preincubated at 37 °C for 5 min with 2 mM ATP (+) and increasing amounts (0.5 and 1 fmol, triangles) of either Dna2 wild type (wt) or Dna2K1080E (KE, helicase, endonuclease) and/or Dna2D657A (DA, helicase, endonuclease; +, 1 fmol; +++, 2 fmol). The reactions were initiated by the addition of MgCl₂ (2 mM) and incubated at 37 °C for an additional 2 min. The reaction products were analyzed on 20% denaturing polyacrylamide gel. M denotes molecular size markers, and the numbers shown on the left of the figure indicate the size of the markers. B, influence of various Dna2 mutants on the viability of Dna2 null cells. The Dna2-deleted strain YKH12 was transformed with the following combinations of plasmids: 1, pRS314-DNA2 and pRS315 (positive control); 2, pRS314 and pRS315 (vectors only control); 3, pRS314-Dna2K1080E and pRS315; 4, pRS314 and pRS315-Dna2D657A; 5, pRS314-Dna2K1080E and pRS315-DNA2D657A. 10-Fold serial dilutions of each transformant were spotted onto complete synthetic media lacking tryptophan, leucine, and histidine in the absence (−FOA) or presence (+FOA) of 5′-fluoroorotic acid. The carbon sources used are indicated.

**FIG. 8.** Uncoupled action of helicase and endonuclease activities of Dna2 in the removal of 3′ end of duplex. The ϕX174 DNA substrate with a 52-bp duplex region is as described in Fig. 4. The standard reaction mixtures (40 µl) containing 15 fmol of substrate and increasing amounts (27.5 and 55 fmol, triangles) of either Dna2 wild type (Dna2wt) or Dna2K1080E (Dna2KE) were incubated at 37 °C for 10 min in the presence (+) or absence (−) of 2 mM ATP and/or 20 ng (110 fmol) of Dna2D657A (Dna2DA). The products were analyzed on 10% polyacrylamide gels. B denotes the boiled substrate control. An arrow indicates the position where the labeled oligonucleotide migrated. The amounts of end label released in the presence of ATP (indicated by an arrowhead) are presented at the bottom of the figure.
Okazaki fragment processing. (i) Dna2 translocates in the 5’ to 3’ direction, consistent with the notion that Dna2 endonuclease cleaves near the junction of a flap starting from its 5’ end. (ii) Dna2 does not require a preformed forked structure to unwind the duplex, in contrast to a previous report (12). Instead, the Dna2 helicase activity is stimulated significantly by a 5’ ssDNA tail but not by a 3’ ssDNA tail. (iii) Dna2 helicase activity is enhanced by RPA as is its endonuclease activity. (iv) Finally, its helicase activity is relatively weak even in the absence of endonuclease activity and acts distributively during the unwinding reaction, in keeping with its catalytic action during Okazaki fragment processing (17).

The presence of the terminal RNA sequence on the 5’-ssDNA neither stimulated nor inhibited the helicase activity, although it activated the endonuclease activity of Dna2 (Fig. 5). At present, it is not clear how the terminal RNA stimulates the endonuclease activity of Dna2 without affecting its helicase activity. Possibly the endonuclease-deficient Dna2D657A binds to an RNA-DNA junction as efficiently as it binds to the 5’-terminal ssDNA end and then unwinds both substrates at the same rate. In contrast, the binding of nucleic-acid-proficient Dna2 to the RNA-DNA junction may directly stimulate the endonuclease activity. The direct stimulation of endonuclease activity by the terminal RNA segment may prevent any unnecessary unwinding by the helicase activity of Dna2. DNA can unwind a DNA-RNA hybrid substrate (data not shown) in which a 20-mer synthetic RNA is annealed internally to a 98-mer DNA template. Such a substrate contained two ssDNA regions at both its 5’ and 3’ ends (30 nt and 25 nt long, respectively). In contrast, substrates such as an RNA-DNA hybrid duplex (in which a 20-mer DNA is internally annealed to a template 98-mer RNA) or RNA-RNA duplex did not support unwinding (data not shown). These findings are consistent with the fact that RNA does not support ATP hydrolysis by Dna2. These results indicate that Dna2 is capable of translocating only along ssDNA but can displace annealed strand.

To explore the role of the DNA helicase activity of Dna2, we examined whether a mixture of Dna2D657A (helicase−, endonuclease−) and Dna2K1080E (helicase−, endonuclease+) behaved similarly to wild type Dna2 in the removal of a flap DNA. The two mutant enzymes, Dna2D657A and Dna2K1080E, when mixed together did not behave similarly to wild type Dna2 (Fig. 7A), indicating that both unwinding and cleavage activities are tightly coupled to each other. Yeast cells expressing these two mutant enzymes in the absence of wild type Dna2 failed to grow. Therefore, the tight coupling of the two enzymatic activities of Dna2 is essential, suggesting that the Dna2 helicase activity facilitates the endonucleolytic cleavage of flap structures. Our data suggest two possible mechanisms by which the helicase activity is utilized for the action of the endonuclease. (i) It may bring Dna2 endonuclease activity near the ssDNA-duplex junction or (ii) it may prevent formation of any secondary structures in the displaced 5’ end region of Okazaki fragments. Furthermore, this process is further assisted by RPA. Based on these results, we conclude that DNA helicase and endonuclease activities of Dna2 are not separable in vivo, at least during Okazaki fragment processing. We cannot rule out the possibility that Dna2 helicase activity is required for some other DNA transactions such as DNA repair.

We observed that the endonuclease-deficient Dna2D657A, which does not cleave flap DNA, reduced the efficiency of Fen1-catalyzed cleavage of a flap substrate, whereas endonuclease-proficient Dna2 did not (data not shown). Therefore, the decreased activity of Fen1 by Dna2D657A (helicase−, endonuclease−) is most likely due to generation of a gap by its ability to displace the flap-containing strand, since Fen1 is...
generally less effective at cleaving these structures (24). This result indicates that the helicase-assisted endonucleolytic cleavage of the flap by Dna2 takes place in a tightly coupled manner, thereby preventing the unnecessary unwinding of the duplex region. In keeping with this, the \textit{in vivo} coexpression of Dna2D657A (helicase$^+$, endonuclease$^-$) and Dna2K1080E (helicase$^-$, endonuclease$^+$) caused the mutant cells to die when cells were grown in media containing glycerol/lactate as a carbon source. These conditions permitted the growth of the Dna2K1080E mutant cells (Fig. 7B; Ref. 21). In summary, Dna2 possesses helicase activity that is well suited to enhancing the endonuclease activity during Okazaki fragment processing.

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Coupling of DNA Helicase and Endonuclease Activities of Yeast Dna2 Facilitates Okazaki Fragment Processing

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