THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 38, Issue of September 22, pp. 22236–22242, 1995
Printed in U.S.A.

Bacteriophage T4 Dda Helicase Translocates in a Unidirectional Fashion on Single-stranded DNA*

(Received for publication, May 4, 1995)

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The T4 bacteriophage Dda helicase is believed to be involved in early events in T4 DNA replication and has been shown to stimulate genetic recombination processes *in vitro*. Dda unwinds double-stranded DNA with 5′ to 3′ polarity but its ability to translocate on DNA has not been established. The DNA stimulated ATPase activity of Dda helicase has been used to probe translocation on single-strand DNA (ssDNA). Dda exhibits higher ATPase activity in the presence of poly(dT) than oligo(dT)$_{16}$, indicating that Dda translocates on ssDNA. Oligonucleotides containing biotin/streptavidin blocks on the 5′ or 3′ end were used to probe directionality of translocation. The K_{act} (K_m for DNA) for Dda ATPase activity was reduced in the presence of a streptavidin block on the 3′ end, whereas a streptavidin block on the 5′ end had only a small effect on the steady-state ATPase parameters. These results suggest that Dda translocates unidirectionally in a 5′ to 3′ manner and upon encountering the block remains bound to the oligonucleotide rather than sliding off the 3′ end. The direction of translocation on ssDNA is consistent with the direction in which Dda unwinds duplex DNA and is not dependent on duplex structure.

Double-stranded (ds)[1] DNA must be melted to provide single strands (ss) for processes such as replication, recombination, and repair. The helicase enzymes responsible for providing ssDNA (Matson and Kaiser-Rogers, 1990; Lohman, 1993) apparently utilize energy available from nucleotide triphosphate hydrolysis to disrupt the molecular interactions which stabilize dsDNA. However, the mechanism(s) by which the helicases transduce chemical energy to mechanical work necessary for dsDNA melting has not been established. Helicase binding to ssDNA, or in some cases dsDNA, stimulates their nucleotide triphosphatase activity, which in turn may drive protein conformational changes required for function (Wong and Lohman, 1992; Chao and Lohman, 1990; Geiselmann *et al.*, 1993).

The mechanism of dsDNA unwinding by the helicase may be related to its mechanism of translocation on ssDNA. Helicases have been categorized by their unwinding directionality, namely whether they unwind in a 5′ to 3′ or 3′ to 5′ fashion, relative to the strand on which they bind. This directional preference observed for dsDNA unwinding implies that helicases may translocate unidirectionally on ssDNA. Although translocation by the enzyme along ssDNA has been shown for a number of helicases, unidirectional translocation on ssDNA has generally not been demonstrated (Young *et al.*, 1994b).

Lohman (Wong and Lohman, 1992) has proposed a rolling mechanism for the *Escherichia coli* Rep helicase mode of action. This enzyme functions as a dimer in which each monomer provides a DNA binding site. Binding of one monomer to ssDNA frees the second to bind and unwind dsDNA at a ss/dsDNA junction. The proposed mechanism for *E. coli* Rep helicase does not necessarily require unidirectional translocation on ssDNA but relies on the polarity of the ss/dsDNA junction to provide directionality in dsDNA unwinding. von Hippel and co-workers (Geiselmann *et al.*, 1993) have proposed a model for *E. coli* Rho helicase in which the enzyme translocates with a 5′ to 3′ directional bias and "unzips" an RNA/DNA hybrid. This mechanism does imply unidirectional translocation by the enzyme on ssRNA. Finally, it is possible that a helicase might unwind dsDNA without translocating via a "stoichiometric" mechanism. In this case, helicase binding near a ss/dsDNA junction induces formation of a region of ssDNA distal from the helicase binding site, to which a second molecule of the helicase may bind. The ss/dsDNA junction may thus be propagated unidirectionally without actual helicase translocation.

The bacteriophage helicase Dda (DNA-dependent ATPase) has been implicated in early events in T4 DNA replication, although its precise role has yet to be determined (Barry and Alberts, 1994; Gauss *et al.*, 1994). Dda has also been shown to stimulate the rate of DNA branch migration catalyzed by the T4 UvsX protein, a recombinase similar in function to *E. coli* Rec A protein (Kodadek and Alberts, 1987). Dda helicase unwinds DNA in a 5′ to 3′ direction in a distributive fashion (Jongeneel *et al.*, 1984) with scant evidence for its translocation on DNA. In this report, the ATPase activity of Dda on different DNA strands has been used as a means to probe the possible translocation of the enzyme along ssDNA. We have constructed DNA strands of varying length as well as DNA strands containing potential blocks to translocation in order to investigate whether this enzyme translocates on ssDNA and, if so, whether translocation is directionally biased.

EXPERIMENTAL PROCEDURES

DNA, Oligonucleotides, and Reagents—Oligonucleotide substrates were synthesized by Operon Technologies (Alameda, CA) and purified by preparative gel electrophoresis. Oligonucleotides containing biotin labels were prepared as described (Kaboord and Benkovic, 1993) and kindly provided by B. F. Kaboord. Poly(dT), poly(dA) and oligonucleotide dT$_{16}$ were purchased from Pharmacia Biotech Inc. Poly(dT) and oligo(dT)$_{16}$ concentrations were measured at 260 nm with an extinction coefficient of 8520 M^{-1} cm^{-1} (Ts'o *et al.*, 1966). For poly(dA), the extinction coefficient was 8600 M^{-1} cm^{-1} at 257 nm (Chamberlin, 1965). Purified oligonucleotides were quantitated by UV absorbance at 260 nm in 0.2 M NaOH using calculated extinction coefficients. Phosphoenolpyruvate (tricyclohexylammonium salt), ATP (disodium salt), NADH, and streptavidin were from Sigma. Phosphoenolpyruvate kinase/lactate dehydrogenase were obtained as a crystalline suspension from Sigma.

Proteins—The Dda helicase was purified following the published

* This work was supported by National Institutes of Health Grants ES05576 (K. D. R.) and GM13306 (S. J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SA, streptavidin; nt, nucleotide.

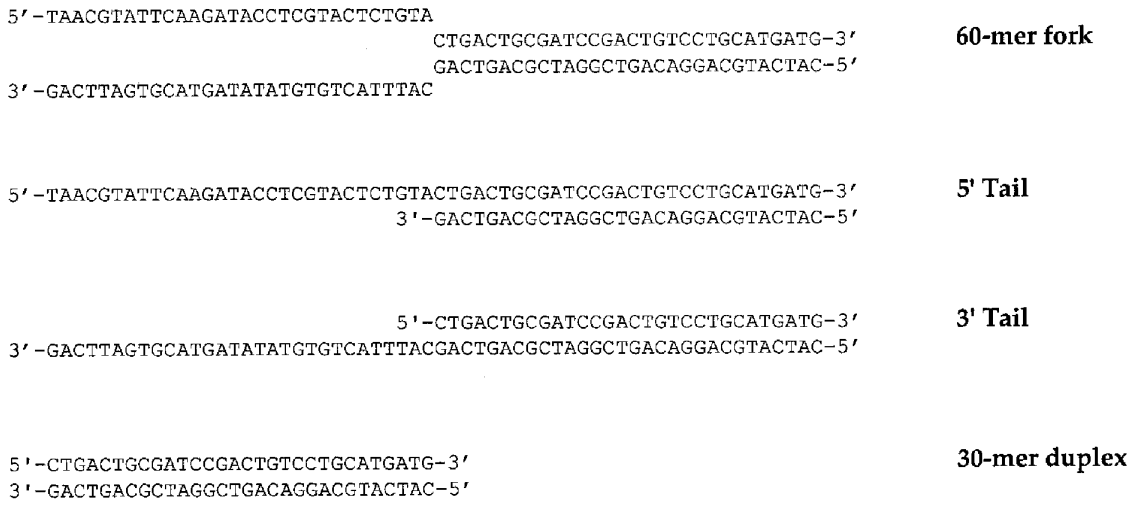


FIG. 1. **Sequences of oligonucleotide substrates for unwinding studies.**

procedure (Hacker and Alberts, 1992) with one exception; the DNase I treatment during cell lysis was eliminated. Dda concentration was determined by UV absorbance in 6 M urea using the extinction coefficient calculated from the amino acid sequence (ϵ_{280} = 59,060 M^{-1} cm^{-1}). T4 32 protein was generously provided by B. F. Kaboord.

ATPase Assays—Dda ATPase activity was monitored spectrophotometrically with the assay described by Panuska and Goldthwait (1980). Except where indicated, all assays were performed at 25 °C in buffer containing 25 mM Tris-OAc, pH 7.5, 10 mM KOAc, 10 mM $Mg(OAc)_2$, 2.5 mM ATP, 1 mM 2-mercaptoethanol, 20 units/ml phosphoenolpyruvate kinase, 29 units/ml lactate dehydrogenase, 4 mM phosphenolpyruvate, 0.9 mM NADH, and 0.1 mg/ml bovine serum albumin. The concentration of Dda for each experiment is indicated in the figure legends as is the concentration of DNA. No difference in steady-state ATPase activity was observed when experiments were initiated by addition of DNA or Dda. The final volume for each reaction mixture was 1 ml. ATP hydrolysis rates were followed by measuring the conversion of NADH to NAD^+ at 380 nm with an extinction coefficient of 1210 M^{-1} cm^{-1}. The oxidation of 1 mol of NADH corresponds to the hydrolysis of 1 mol of ATP.

DNA Unwinding Assays—Unwinding experiments were carried out using a native polyacrylamide gel assay (Venkatesan *et al.*, 1982: Matson and Kaiser-Rogers, 1990). The leading strand of the substrate was labeled with ^{32}P and annealed to the appropriate complementary strand to create the desired substrate. Unreacted [γ-^{32}P]ATP was removed by passing the labeled DNA through two Sephadex G-25 spin columns. The labeled DNA was then added to a stock solution of unlabeled substrate. DNA was added to the helicase assay buffer and unwinding was initiated by adding Dda. The final concentration of reagents in the unwinding reactions was 25 mM Tris-OAc, pH 7.4, 10 mM $Mg(OAc)_2$, 10 mM KOAc, and 3 mM ATP in a final volume of 20 μl. DNA and Dda concentrations are shown in the figure legends. Reactions were stopped after 10 s by adding 20 μl of quench solution containing 0.5 M EDTA and a 30-mer oligonucleotide identical in sequence to the duplex region of the labeled strand of the substrate. The latter served as a trap to prevent labeled product from reannealing with its complementary strand. Final concentration of the 30-mer trapping strand was 20 times higher than the concentration of the substrate. Nondenaturing load buffer (5 μl, 50% glycerol, v/v) was added to 10 μl of each quenched reaction mixture, and samples were electrophoresed on a native 15% polyacrylamide gel. Gel images were obtained and analyzed using a Molecular Dynamics PhosphorImager. The amount of unwinding was determined by comparing relative amounts of ssDNA and dsDNA with corrections for trap efficiency and ssDNA in control samples.

Fluorescence Titrations—The intrinsic fluorescence of Dda helicase is quenched upon binding to DNA. Titrations of Dda with DNA were carried out using an SLM Aminco spectrofluorometer at 25 °C in 25 mM Tris-OAc, pH 7.4, 150 mM KOAc, and 1 mM 2-mercaptoethanol. Correction for dilution during the titration was accomplished by titrating a sample containing only tryptophan in parallel with the Dda titration. The excitation wavelength for Dda was 281 nm and the emission wavelength 341 nm.

RESULTS

Dda Helicase Unwinding Activity Exhibits 5′ to 3′ Polarity—Unwinding experiments were carried out to extend previous observations which indicated that Dda unwinds dsDNA with a 5′ to 3′ polarity with respect to the strand to which Dda is bound (Jongeneel *et al.*, 1984). The purified oligonucleotides were annealed to construct the four different substrates used for unwinding studies (Fig. 1). DNA unwinding was carried out using a gel assay in which the ssDNA product was trapped after quenching the reaction by adding an excess of an unlabeled strand which prevents reannealing of product (Raney *et al.*, 1994). The ssDNA was separated from dsDNA on a native polyacrylamide gel with the relative quantities of ssDNA and dsDNA determined by quantitation of the radioactivity in each band using a Molecular Dynamics PhosphorImager. The results are shown in Table I.

The 60-mer fork and 5′ tail substrates were unwound to a similar degree under these conditions. However, the 3′ tail and 30-mer duplex substrates were not measurably denatured during the 10-s reaction time. Thus, Dda unwinds DNA with a 5′ to 3′ polarity as previously shown but does not unwind a blunt end duplex oligonucleotide. The fact that the unwinding is directional suggests that the helicase may translocate in a 5′ to 3′ direction on ssDNA. This presumption is not necessarily valid (Young *et al.*, 1994b). Translocation on ssDNA might not be directionally biased, if unwinding is unidirectional only as a consequence of the ss/dsDNA junction inducing the directionality (Lohman, 1993). Since Dda unwinds dsDNA in a distributive fashion, rapidly dissociating from DNA during the progress of the reaction, this helicase may not translocate at all (Raney *et al.*, 1994; Jongeneel *et al.*, 1984; Krell *et al.*, 1979). Direct evidence for Dda translocation on ssDNA is required.

Dda Binding to Poly(dA) Measured by ATPase Activity and Fluorescence Quenching—Dda binding to poly(dA) was investigated by two methods. The stimulation of its ATPase activity as a function of increasing poly(dA) concentration gave rise to a saturation curve (Fig. 2*A*). The K_{act} determined for poly(dA) under these conditions is 31 ± 1.8 μM nt. From the intrinsic fluorescence quenching which occurs upon binding of Dda to poly(dA) (Fig. 2*B*), a K_d of 8.0 ± 1.0 μM nt poly(dA) was obtained. The difference in poly(dA) concentration required for Dda saturation in these two experiments indicates a possible difference in DNA binding by the enzyme in the presence of ATP hydrolysis. The difference is small and more compelling evidence was sought for translocation by measuring ATPase

TABLE I
DNA substrate specificity of Dda helicase

Unwinding of various DNA substrates was measured as described under "Experimental Procedures." Reaction mixtures contained 250 nM Dda in 25 mM Hepes, pH 7.4, 10 mM $Mg(OAc)_2$, and 2.5 mM ATP.

Oligonucleotide	Substrate concentration	Product
	nM	
DNA fork	200	112
5′ tail	200	120
DNA fork	100	66
3′ tail	100	<2[a]
30-mer duplex	100	<2[a]

[a] No product was detected during the 10-s reaction time (2 nM detection limit).

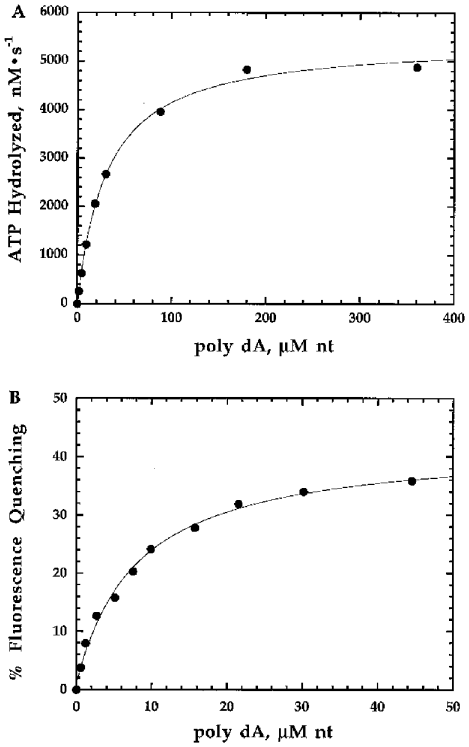
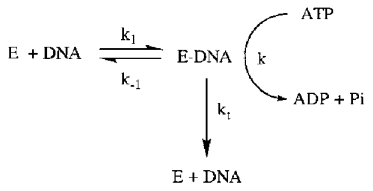


FIG. 2. **Binding of Dda helicase with poly(dA).** Binding of Dda to poly(dA) was investigated by fluorescence quenching and by ATPase activity. *A*, ATPase activity was measured as a function of poly(dA) using the coupled ATPase assay described under "Experimental Procedures." Dda concentration was 50 nM, and KOAc concentration was 160 mM. The K_{act} of 31 ± 1.8 μM nt was obtained by fitting the data to the Michaelis-Menten equation. *B*, fluorescence titration of Dda (150 nM) with poly(dA) in 25 mM Tris-OAc, pH 7.5, 160 mM KOAc at 25 °C. The data were fit to the quadratic equation, yielding a K_d of 8 ± 1.0 μM nt.

activity on DNA substrates of varying length and/or containing potential blocks to translocation.

A simple kinetic model (Scheme I) describing steady-state ATPase activity for ATP-driven translocases has been proposed (Young *et al.*, 1994a). The translocation process is represented by k_t, which is the rate constant for dissociation of the enzyme from the end of the DNA lattice via translocation and is dependent on DNA length. All enzyme-DNA species which are capable of hydrolyzing ATP are combined into one term, *E*·DNA, which hydrolyzes ATP with the rate constant k. The terms describing V_{max} (Equation 1, e is the total enzyme concentration) and K_{act} (the K_m for DNA, Equation 2) for this simple model predict that increasing DNA length should result in decreased values of K_{act} while not changing V_{max}. This model provides a useful context in which to analyze DNA dependent ATPase activity.



SCHEME I.

$$V_{max} = ek \quad \text{(Eq. 1)}$$

$$K_{act} = \frac{k_t + k_{-1}}{k_1} \quad \text{(Eq. 2)}$$

ATPase Activity on Varying Length Oligonucleotides—Several groups have shown a length dependence for the DNA stimulation of helicase ATPase activity (Young *et al.*, 1994b; Matson and George, 1987; Liu and Alberts, 1981). A translocating helicase is expected to show differential stimulation of ATPase activity as a function of DNA length due to the helicase advancing to the ends of shorter DNA fragments more quickly than longer ones. No DNA length dependence has been shown for Dda ATPase stimulation, since ATPase activity seen with a 10-mer oligonucleotide was the same as with longer polynucleotides (Jongeneel *et al.*, 1984; Krell *et al.*, 1979). In our hands, oligonucleotides of different lengths likewise showed only small differences in their stimulation of Dda ATPase activity (Fig. 3, Table II). The shortest oligonucleotide, 13-mer, was the most effective; the longest oligonucleotide, 50-mer, was the least stimulatory, suggesting that the helicase was sensitive to the particular DNA sequence as well. The observation that a 13-mer can support ATPase activity is consistent with the helicase binding site size of 13 nt or less. The minimal length necessary for unwinding of dsDNA by Dda is a 12-nt ssDNA overhang (Krell *et al.*, 1979). The actual binding site size awaits determination of the oligomeric nature of Dda, which can affect estimates of this value (Runyon *et al.*, 1993). Since the differences in stimulation of Dda ATPase activity by various lengths of DNA are small and sequence sensitive, we next compared two polymers of identical sequence and greatly differing length.

Dda ATPase Activity Is Dependent on DNA Length—Poly(dT) and a short homopolymer, $oligo(dT)_{16}$, were compared for their ability to support Dda ATPase stimulation. A clear difference in their ability to stimulate Dda ATPase activity was observed (Fig. 4). The K_{act} values for poly(dT) and $oligo(dT)_{16}$ were 300 nM nt and 690 nM nt, V_{max} values were 2660 $nM \cdot s^{-1}$ and 1920 $nM \cdot s^{-1}$, respectively. Young *et al.* (1994b) reported a similar phenomenon for the T4 41 helicase, although differences in K_{act} were not observed for lengths above 50 nt. The difference in the K_{act} values suggests that Dda translocates on ssDNA, since the lower value is seen with the longer strand as expected for a translocating helicase (Equation 2). The change in V_{max} for poly(dT) compared with $oligo(dT)_{16}$ suggests that the simple translocation model in Scheme I is inadequate for describing Dda ATPase activity. A second, kinetically distinct, step is required to account for the results in Fig. 4, and this mechanism is shown in Scheme II. The species [*E*·DNA]′ represents the Dda helicase that hydrolyzes ATP with rate constant k' while translocating along poly(dT). The second species [*E*·DNA]″ represents the helicase bound to DNA at the end of the lattice and hydrolyzes ATP with rate constant k''. The term describing dissociation from the end of the DNA lattice is k_d, whereas k_t is the rate constant for translocation to the end of the lattice.

This mechanism is similar to that outlined by Young *et al.* (1994a), with the difference being that the enzyme species [*E*·DNA]″ in Scheme II can hydrolyze ATP, whereas a similar

13-mer 5'-TCGCAGCCGTCCA-3'

23-mer 5'-CAAAAATTACGTGCGGAAGGAGT-3'

34-mer 5'-ACTCCTTCCGCACGTAATTTTTGACGCACGTTGT-3'

50-mer 5'-CTTACTGACGCATCAGACAACGTGCGTCAAAAATTACGTGCGGAAGGAGT-3'

FIG. 3. **ATPase activity on varying length oligonucleotides.** Sequences of oligonucleotides used to investigate the dependence of Dda ATPase activity on DNA length.

TABLE II
Dda ATPase activity stimulated by oligonucleotides

Dda ATPase activity was measured using the coupled assay system described under "Experimental Procedures." The sequences of the oligonucleotides are shown in Fig. 3. The K_{act} and V_{max} values were obtained by fitting the data to the Michaelis-Menten equation.

Oligonucleotide	K_{act}	V_{max}
	μM *nt*	$nM \cdot s^{-1}$
13-mer	1.8 ± 0.2	3136 ± 74
23-mer	6.7 ± 0.5	3270 ± 75
34-mer	3.8 ± 0.5	3129 ± 109
50-mer	17.8 ± 2.2	3636 ± 130

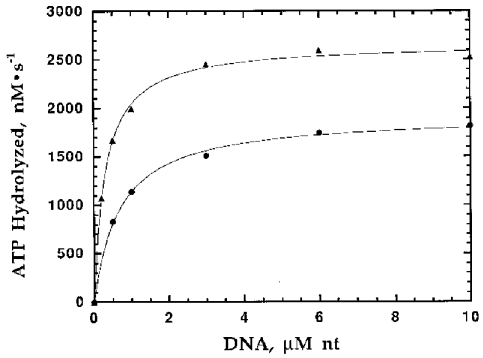
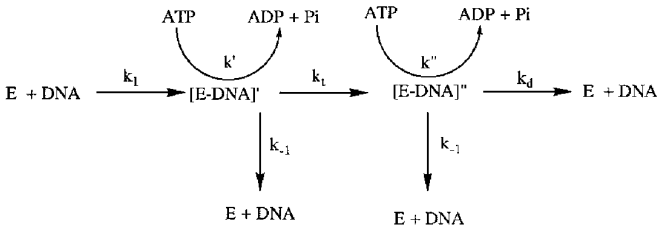


FIG. 4. **Dda ATPase activity is dependent on substrate length.** ATPase activity was measured in the presence of varying concentrations of poly(dT) (▲) and oligo(dT)$_{16}$ (●) using a Dda concentration of 25 nM. Data were fit to the Michaelis-Menten equation (*solid lines*). The V_{max} and K_{act} values for poly(dT) were 2660 $nM \cdot s^{-1}$ and 300 nM nt, whereas V_{max} and K_{act} for oligo(dT)$_{16}$ were 1920 $nM \cdot s^{-1}$ and 690 nM nt, respectively.



SCHEME II.

species described by Young could not hydrolyze ATP. The resulting terms describing V_{max} and K_{act} are shown in Equations 3 and 4, respectively.

$$V_{max} = \left[\frac{k'(k_d + k_{-1}) + k''k_t}{k_t + k_d + k_{-1}}\right]e \quad \text{(Eq. 3)}$$

$$K_{act} = \frac{(k_t + k_{-1})(k_d + k_{-1})}{k_1(k_t + k_d + k_{-1})} \quad \text{(Eq. 4)}$$

The rate constant which is dependent on DNA length, k_t, now appears in both the V_{max} and K_{act} terms, indicating that both kinetic parameters can be affected by DNA lattice length, as seen in Fig. 4.

ATPase Activity on ssDNA, DNA Fork Substrates, and ssDNA Bound by T4 32 Protein—Helicase translocation on DNA may be impeded by steric features within the substrate that result in changes in the helicase's ATPase activity. One potential alteration is a region of dsDNA adjacent to a ssDNA region. The ATPase activity by a helicase on ssDNA may differ from that which occurs during dsDNA unwinding. T7 gene 4 helicase exhibited lower ATPase activity on a 75-mer oligonucleotide containing a short duplex region on the 3′ end, when compared with the 75-mer alone (Notarnicola and Richardson, 1994). A short dsDNA region on the 5′ end produced no reduction in ATPase activity. These results are in accord with the unidirectional translocation of the gene 4 helicase on ssDNA. On the other hand, no change in ATPase activity was observed for T4 41 helicase when comparing M13 ssDNA with and without short oligonucleotides capable of forming dsDNA regions (Young *et al.*, 1994b). Finally, RecBCD helicase hydrolyzes ATP more rapidly during unwinding of dsDNA than in the presence of ssDNA (Roman and Kowalczykowski, 1989). Thus, relative rates of hydrolysis of ATP during ssDNA binding and dsDNA unwinding reflect the properties of the helicase and may provide, in some cases, a means to study unidirectional translocation on ssDNA.

The ATPase activity of Dda was measured using a DNA fork substrate to determine if any difference existed in stimulation of ATPase activity during unwinding of dsDNA as compared with binding to ssDNA, Fig. 5. Previous work (Raney *et al.*, 1994) had shown that Dda unwinds the 60-mer fork until the unwinding rate and reannealing rate of product reach a steady state (~50% when Dda is 50 nM and the 60-mer fork is 250 nM). Thus, the ATPase activity measured under steady-state conditions represents Dda ATPase activity that is stimulated partially by unwinding of the 60-mer fork DNA and partially by binding to ss 60-mer. ATPase activity is stimulated very little by blunt end dsDNA, since a 30-mer duplex at 250 nM gave rise to ATPase activity of 154 $nM \cdot s^{-1}$, whereas the ssDNA 60-mer at the same concentration produced a rate of 3400 $nM \cdot s^{-1}$ (data not shown). The ATPase data in Fig. 5 is plotted in terms of the concentration of 60-mer strands in order to compare directly the quantity of DNA used in each experiment (see Fig. 1 for sequences of the 60-mer and the 60-mer fork substrates). The data provided identical K_{act} values for each substrate of 150 nM strands, whereas V_{max} for the ssDNA 60-mer was slightly higher than the 60-mer fork, 4940 $nM \cdot s^{-1}$ *versus* 3890 $nM \cdot s^{-1}$. No conclusion can be drawn with regard to translocation directionality of the Dda helicase, except that the enzyme's ATPase activity is insensitive to the single strand or duplex nature of the DNA fork substrate.

Finally, T4 32 single strand-binding protein was bound to ssDNA in order to impede either binding and/or translocation of the Dda helicase. Data from the inhibition of T4 41 helicase ATPase activity by T4 32 single strand-binding protein has been successfully analyzed in terms of such a model (Young *et al.*, 1994b). Dda ATPase activity was measured using poly(dT) and varying concentrations of 32 with the results being as shown in Fig. 6. At low 32 concentration, a small but reproducible enhancement of Dda ATPase activity occurs. Inhibition

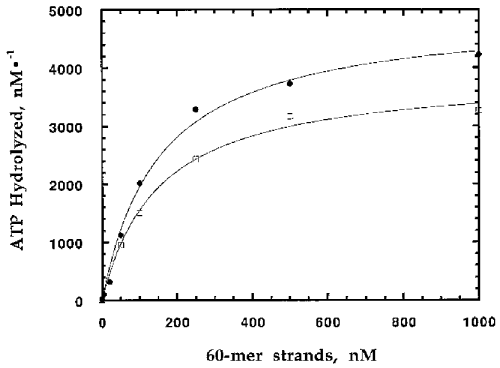


FIG. 5. **Dda ATPase activity on single strand 60-mer and partial duplex 60-mer fork substrates.** ATPase activity was measured as a function of concentration of ssDNA 60-mer strands (●) and a partially duplex 60 mer fork molecules (□). For comparison, results are shown in terms of concentration of 60-mer strands, *i.e.* one 60-mer fork contains two 60-mer strands. Dda concentration was 50 nM. The V_{max} and K_{act} for the 60-mer ssDNA were 4940 nM·s^{-1} and 150 nM strands, whereas V_{max} and K_{act} for the 60-mer fork were 3890 nM·s^{-1} and 150 nM strands, respectively.

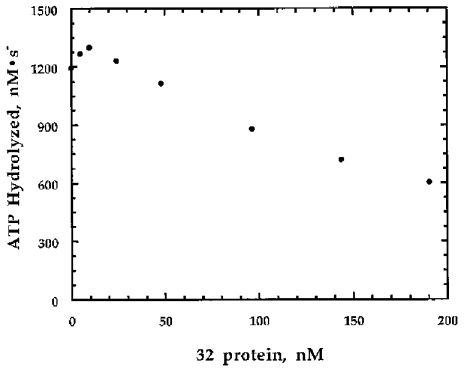


FIG. 6. **Inhibition of Dda ATPase activity by the T4 gene 32 single strand-binding protein.** ATPase activity was measured with 25 nM Dda, 500 nM poly(dT), and varying amounts of 32 protein. The concentration of 32 protein necessary to completely cover the DNA is 71 nM, assuming a site size of 7 nt for 32 protein (Kowalczykowski *et al.*, 1981). Dda ATPase activity is only partially inhibited, indicating that Dda is able to compete with 32 for DNA under these conditions.

ensues at higher levels of 32, but the degree of inhibition does not follow that expected if 32 is preventing Dda from binding. The concentration of 32 necessary to completely cover the DNA is 71 nM based on a 7-nucleotide site size for 32 (Kowalczykowski *et al.*, 1981). Inhibition at this concentration is only ~30% which agrees qualitatively with previous reports (Jongeneel *et al.*, 1984). The degree of inhibition is insufficient to pursue the analysis described by Young *et al.* (1994b).

Dda ATPase Activity on Biotinylated Oligonucleotides—The 50-mer oligonucleotide was assembled by ligation of a 23- and 27-mer as described in Kaboord and Benkovic (1993). For the biotin-labeled 50-mers, the 23-mer contained a 3′-biotin and the 27-mer contained a 5′-biotin (Fig. 7*A*). The 3′ or 5′-biotin-labeled 50-mers were used as substrates for stimulation of Dda ATPase activity. Streptavidin (SA) when bound to biotin would serve as blocks at the ends of the oligonucleotide. We reasoned that if Dda translocates, it would encounter the streptavidin block at an end of the oligonucleotide, and its ATPase activity might be altered. Thus, placing the block on either end of the 50-mer might provide evidence for a strong directional bias in Dda translocation on ssDNA.

The overall effect that this change may have on V_{max} and K_{act} is difficult to evaluate due to the complexity of Equations 3 and 4. However, for an oligonucleotide, one reasonable assumption is that k_t, the rate constant for translocation to the end of the DNA lattice, is large relative to k_{-1} and k_d. This is so because the lattice is very short, and k_t is length-dependent. Thus, the K_{act} term collapses to Equation 5, and the term for V_{max} collapses to Equation 6.

$$K_{act}^{oligo} = \frac{k_d + k_{-1}}{k_1} \quad \text{(Eq. 5)}$$

$$V_{max}^{oligo} = \left[k' \left(\frac{k_d + k_{-1}}{k_t} \right) + k'' \right] e \quad \text{(Eq. 6)}$$

Placement of a block which prevents translocation from the end of the DNA effectively reduces k_d to 0, thus the K_{act} term reduces further to Equation 7 in the presence of the SA block.

$$K_{act}^{oligo\text{-}block} = \frac{k_{-1}}{k_1} \quad \text{(Eq. 7)}$$

$$V_{max}^{oligo\text{-}block} = \left[k' \left(\frac{k_{-1}}{k_t} \right) + k'' \right] e \quad \text{(Eq. 8)}$$

Comparison of Equations 5 and 7 enables a clear prediction that the K_{act} will be reduced in the presence of the SA block. Comparison of V_{max} terms (Equations 6 and 8) indicates that this parameter is not expected to change in the presence of the block.

Both the 3′-biotinylated and unlabeled 50-mer supported ATPase activity in the absence of streptavidin (Fig. 7*B*, Table II). When streptavidin was added, the K_{act} for ATPase activity was reduced (Fig. 7*B*). The data in Fig. 7*B* provided a K_{act} of 19.8 μM nt for the 3′-biotin 50-mer in the absence of SA and a K_{act} of 2.3 μM nt in the presence of SA. The large difference observed in the K_{act} values suggests that the Dda is sequestered on the 50-mer, resulting in an increase in the residence time of Dda on the oligonucleotide in the presence of SA, as predicted if k_d were effectively eliminated. The lack of a significant effect on V_{max} in the presence of the block is also consistent with the kinetic model (Equations 6 and 8). The 5′-biotin 50-mer stimulated Dda ATPase activity to a similar degree as did the 3′-biotin 50-mer and the unlabeled 50-mer. The addition of SA to the 5′-biotin 50-mer had little effect on the Dda ATPase activity (Fig. 7*C*). The contrasting reduction in K_{act} for the 3′-biotin 50-mer in the presence of SA *versus* the lack of a similar effect for the 5′-biotin 50-mer suggests that Dda translocates in a 5′ to 3′ direction on ssDNA.

We sought to determine whether the increase in ATPase activity on the 3′-biotin 50-mer in the presence of SA was due to a structural effect in the 50-mer oligonucleotide that was removed in the presence of SA. The 23-mer from which the 50-mer was constructed was found to support ATPase activity better than the 50-mer, and thus the 3′-biotin-labeled form of this oligonucleotide was used as a substrate for measuring ATPase activity. The results are shown in Table III along with results for the 5′-biotin 27-mer. The same trend was observed with these substrates at low DNA concentration, as was seen with the biotin-labeled 50-mers. The effects on Dda ATPase activity observed upon SA binding to the biotinylated oligonucleotides are therefore not due to a structural anomaly in the 50-mer. The low ATPase activity found with the 50-mer is probably due to a sequence effect contained within the 27-mer which also stimulated Dda ATPase activity poorly (Table III).

DISCUSSION

Helicases show a preference for unwinding a dsDNA possessing a flanking ssDNA region that serves as an initiation site for the unwinding reaction. The actual substrate may have the ssDNA as 5′ to 3′ or 3′ to 5′ with respect to the duplex with the preferred substrate bound determining the polarity of the helicase. The requirement of many helicases for a region of

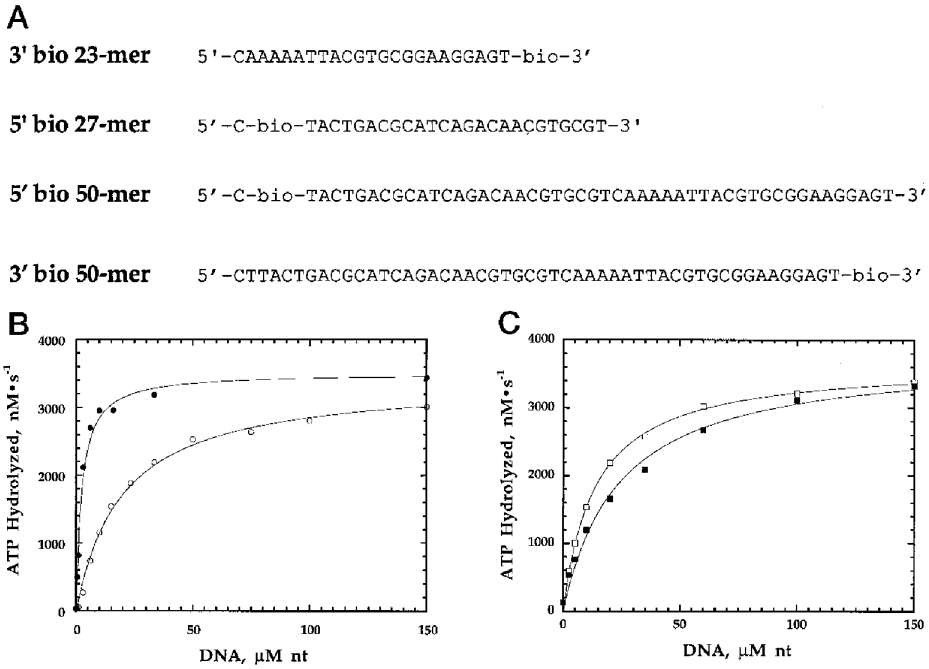


FIG. 7. **Effect of biotin/streptavidin blocks on Dda ATPase activity.** ATPase activity of 25 nM Dda was measured in the presence of the 50-mer oligonucleotide containing a biotin label on either the 5′ or 3′ end. *A*, sequences of biotin-labeled oligonucleotides. *B*, Dda ATPase activity as a function of the 3′-biotin-labeled 50-mer in the presence (●) or absence (○) of 560 nM streptavidin. The K_{act} determined for this substrate was 19.8 ± 1.5 μM nt in the absence of SA and 2.3 ± 0.3 μM nt in the presence of SA. V_{max} values in the presence and absence of SA were 3420 ± 80 nM·s^{-1} and 3510 ± 100 nM·s^{-1}, respectively. *C*, Dda ATPase as a function of the 5′-biotin-labeled 50-mer in the presence (■) or absence (□) of 560 nM streptavidin. K_{act} values obtained in the presence and absence of SA were 24.0 ± 3.0 μM nt and 13.7 ± 1.0 μM nt, respectively and V_{max} values in the presence and absence of SA were 3791 ± 147 nM·s^{-1} and 3665 ± 52 nM·s^{-1}, respectively.

TABLE III
Dda ATPase activity with biotin-labeled oligonucleotides

Dda ATPase activity was measured at 3 μM nucleotide and 25 nM Dda for each oligonucleotide in the assay buffer described under "Experimental Procedures."

Substrate	ATP Hydrolyzed	
	− streptavidin	+ streptavidin
	nM·s^{-1}	
50-mer	220	220
5′-Biotin 50-mer	377	240
3′-Biotin 50-mer	272	2120
3′-Biotin 23-mer	410	1057
5′-Biotin 27-mer	129	90

ssDNA implies an association of translocation on ssDNA with unwinding of dsDNA and that the two processes may share a common mechanism. One mechanism for translocation of the *E. coli* Rep helicase (Wong and Lohman, 1992; Amaratunga and Lohman, 1993) is based on a dimer state for the helicase with two DNA binding sites that alternatively bind to ssDNA or dsDNA in a rolling fashion. The ss/dsDNA junction provides the polarity which ensures unidirectional translocation through dsDNA, but not necessarily ssDNA. Others have proposed that the *E. coli* Rho helicase (Geiselmann *et al.*, 1993), *E. coli* UvrD helicase (Matson, 1986), T7 gene 4 helicase (Notarnicola and Richardson, 1994), and T4 41 helicase (Young *et al.*, 1994b) translocate undirectionally along ssRNA or ssDNA.

The question of translocation on ssDNA for the T4 Dda helicase is addressed here. Previous work (Raney *et al.*, 1994; Jongeneel *et al.*, 1984; Krell *et al.*, 1979) has indicated that Dda acts distributively, rapidly dissociating from DNA, making the directionality of translocation difficult to investigate with this enzyme. The precise function of Dda *in vivo* is not known; however it is believed to be involved in recombination and in early events in replication (Barry and Alberts, 1994; Gauss *et al.*, 1994). The activity of Dda is not essential and can be replaced by the T4 41 helicase in conjunction with the T4 59 protein, although T4 strains which are both Dda$^-$ and 59$^-$ are not viable (Gauss *et al.*, 1994).

We sought to determine whether the 5′ to 3′ polarity observed in dsDNA unwinding by Dda (Table I) is also reflected in the enzyme's translocation on ssDNA. No length dependence for the DNA stimulation of Dda ATPase activity has been demonstrated previously (Jongeneel *et al.* 1984; Krell *et al.*, 1979). Thus, Dda might operate by a stoichiometric mechanism whereby one molecule of Dda bound near the ss/dsDNA junction could induce local melting of the dsDNA providing a region of ssDNA. A second molecule of Dda could then bind to the newly exposed ssDNA, and this process could lead to directional unwinding of dsDNA without translocation of the helicase.

With varying length oligonucleotides (Table II), no pattern for the effect of DNA length on the kinetic parameters, K_{act} and V_{max}, was observed. Since DNA sequence may be important for this helicase, at least with oligonucleotides, we compared DNA-stimulated ATPase activity in the presence of poly(dT) and oligo(dT)$_{16}$ (Fig. 4) and observed a clear difference in Dda ATPase activity. The length-dependent stimulation of helicase ATPase activity was rationalized by a scheme featuring two species capable of hydrolyzing ATP. The first includes all forms of *E*·DNA′ where the enzyme is within the substrate lattice; the second (*E*·DNA″) represents helicase bound at the ssDNA terminus (Scheme II). Both species are assigned different turnover numbers for ATP hydrolysis with their relative concentrations reflecting the rate of conversion via k_t of *E*·DNA′ to *E*·DNA″ by translocation of the helicase. This linkage explicitly introduces the dependence on ssDNA length of the ATPase activity.

We then sought data for the translocation directionality of the helicase on ssDNA by using substrates containing potential translocation blocks. The T4 32 ssDNA-binding protein had been previously used to inhibit T4 41 ATPase activity and, presumably, translocation of the helicase (Young *et al.*, 1994b). Our attempt to apply this method to Dda helicase indicated that ATPase activity on poly(dT) is not completely inhibited by 32 and may in fact be stimulated at low 32 concentrations (Fig. 6), consistent with a possible functional interaction between Dda and T4 32 protein (Formosa *et al.*, 1983). We therefore sought a different protein block to inhibit Dda translocation. Some protein blocks do not inhibit Dda unwinding, suggesting that in these cases, Dda is capable of translocating through the block (Bedrosian and Bastia, 1991; Yancey-Wrona and Matson, 1992). Maine and Kodadek (1994) prepared a partial duplex DNA substrate containing a binding site for GAL4 and reported the reduction in ATPase activity and DNA unwinding by

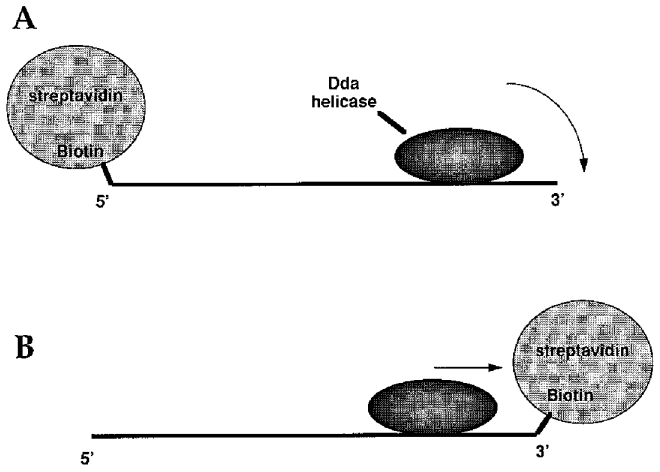


FIG. 8. **Diagram depicting Dda helicase translocation on biotin/streptavidin labeled oligonucleotides.** *A*, 5′ to 3′ unidirectional translocation leads to rapid dissociation from the DNA end of the 5′-biotin-labeled oligonucleotide. *B*, dissociation from the end of the 3′-biotin-labeled oligonucleotide is blocked in the presence of streptavidin. Dda is effectively sequestered onto the DNA and K_{act} is lowered.

Dda in the presence of the GAL4·DNA complex. However, this approach does not distinguish between unidirectional and bidirectional translocation on ssDNA.

Thus, we prepared two ssDNA substrates containing an asymetrically located protein block which would not be displaced by Dda. Two 50-mer oligonucleotides with a biotin label covalently attached on either the 5′ or 3′ end were constructed, that when bound to SA, provided a translocation block which was unlikely to be displaced (Fig. 7*A*). A similar substrate was effective in the reconstitution of the T4 polymerase holoenzyme by preventing the T4 45 protein sliding clamp from dissociating from end of the DNA during assembly of the holoenzyme complex (Kaboord and Benkovic, 1993).

In the absence of SA, these substrates had little or no effect on the stimulation of the ATPase activity of Dda (compare Fig. 7, *B* and *C*, with Table II). However, in the presence of SA, a reduction in K_{act} for Dda ATPase activity was observed only with the 3′-biotin labeled 50-mer (Fig. 7*B*). The 8-fold reduction in K_{act} for this substrate implies that Dda does not dissociate as rapidly from this DNA strand due to the SA block, consistent with SA acting to prevent translocation from the 3′ end of the oligonucleotide and thereby effectively eliminating k_d in Scheme II. In contrast, the addition of SA to the 5′-biotin-labeled 50-mer led to only a small increase in the K_{act} for Dda ATPase activity (Fig. 7*C*).

We interpret these results to indicate that Dda translocates unidirectionally on ssDNA in a 5′ to 3′ direction. The effect of SA at the 3′ end is to sequester Dda onto the DNA, preventing rapid dissociation of the helicase from the end of the DNA (Fig. 8). One might expect that upon halting helicase translocation, the V_{max} for ATPase activity would also be reduced, although the results described here indicate that no reduction has occurred. This observation suggests that enzyme translocation and ATP hydrolysis may have been decoupled. However, Dda may rebound from the SA block and continue to catalyze the hydrolysis of ATP. If this helicase, like all other DNA helicases studied to date, exists in an oligomeric form, then it should contain multiple DNA binding sites which can bind DNA in an ordered fashion coupled to ATP binding and hydrolysis. Thus, while translocation from the end of the DNA is inhibited, the cycle of DNA binding events proposed to occur with helicase activity and associated ATP hydrolysis may continue. The recent observation that the *E. coli* RuvB helicase binds to DNA by encircling the DNA (Stasiak *et al.*, 1994) provides an attractive means for a helicase to remain topologically bound to DNA while being inhibited from translocating due to the SA block, although the oligomeric nature of Dda has not been established. We have observed the same phenomenon with the T4 41 helicase as well; *i.e.* the ATPase activity of T4 41 helicase is enhanced by the 3′ SA block at low DNA concentration.[2]

Acknowledgments—We thank Dr. Barbara F. Kaboord for providing biotinylated oligonucleotides, Dr. Theodore E. Carver for invaluable discussions, and Patricia Benkovic for critical reading of the manuscript.

REFERENCES

Amaratunga, M., and Lohman, T. M. (1993) *Biochemistry* **32,** 6815–6820
Barry, J., and Alberts, B. (1994) *J. Biol. Chem.* **269,** 33063–33068
Bedrosian, C. L., and Bastia, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2618–2622
Chao, K., and Lohman, T. M. (1990) *J. Biol. Chem.* **265,** 1067–1076
Chamberlin, M. C. (1965) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **24,** 1446–1457
Formosa, T., Burke, R. L., and Alberts, B. M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2442–2446
Gauss, P., Park, K., Spencer, T. E., and Hacker, K. J. (1994) *J. Bacteriol.* **176,** 1667–1672
Geiselmann, J., Wang, Y., Seifried, S. E., and von Hippel, P. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7754–7758
Hacker, K. J., and Alberts, B. M. (1992) *J. Biol. Chem.* **267,** 20674–20681
Jongeneel, C. V., Formosa, T., and Alberts, B. M. (1984) *J. Biol. Chem.* **259,** 12925–12932
Kaboord, F. B., and Benkovic, S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10881–10885
Kodadek, T., and Alberts, B. M. (1987) *Nature* **326,** 312–314
Kowalczykowski, S. C., Lonberg, N., Newport, J. W., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145,** 75–104
Krell, H., Dürwald, H., and Hoffman-Berling, H. (1979) *Eur. J. Biochem.* **93,** 387–395
Liu, C.-C., and Alberts, B. M. (1981) *J. Biol. Chem.* **256,** 2813–2820
Lohman, T. M. (1993) *J. Biol. Chem.* **268,** 2269–2272
Maine, I. P., and Kodadek, T. (1994) *Biochem. Biophys. Res. Commun.* **198,** 1070–1077
Matson, S. M. (1986) *J. Biol. Chem.* **261,** 10169–10175
Matson, S. W., and George, J. W. (1987) *J. Biol. Chem.* **262,** 2066–2076
Matson, S. W., and Kaiser-Rogers, K. A. (1990) *Annu. Rev. Biochem.* **59,** 289–329
Notarnicola, S. M., and Richardson, C. C. (1994) *J. Cell. Biochem.* **18C,** 153
Panuska, J. R., and Goldthwait, D. A. (1980) *J. Biol. Chem.* **225,** 5208–5214
Raney, K. D., Sowers, L. C., Millar, D. P., and Benkovic, S. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6644–6648
Roman, L. J., and Kowalczykowski, S. (1989) *Biochemistry* **28,** 2873–2881
Runyon, G. T., Wong, I., and Lohman, T. M. (1993) *Biochemistry* **32,** 602–612
Stasiak, A., Tsaneva, I. R., West, S. C., Benson, C. J. B., Yu, X., and Egelman, E. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7618–7622
Ts'o, P. O. P., Raport, S. A., and Bollum, F. J. (1966) *Biochemistry* **5,** 4153–4170
Venkatesan, M., Silver, L. L., and Nossal, N. G. (1982) *J. Biol. Chem.* **257,** 12426–12434
Wong, I., and Lohman, T. M. (1992) *Science* **256,** 350–355
Yancey-Wrona, J. E., and Matson, S. W. (1992) *Nucleic Acids Res.* **20,** 6713–6721
Young, M. C., Kuhl, S. B., and von Hippel, P. H. (1994a) *J. Mol. Biol.* **235,** 1436–1446
Young, M. C., Schultz, D. E., Ring, D., and von Hippel, P. H. (1994b) *J. Mol. Biol.* **235,** 1447–1458

[2] K. D. Raney and S. J. Benkovic, unpublished observations.