DnAB from *Thermus aquaticus* Unwinds Forked Duplex DNA with an Asymmetric Tail Length Dependence*

(Received for publication, August 28, 1998, and in revised form, November 9, 1998)

Daniel L. Kaplan‡§ and Thomas A. Steitz‡¶**

*From the Departments of Molecular Biophysics and Biochemistry and Chemistry, Yale University and the Howard Hughes Medical Institute, New Haven, Connecticut 06520-8114*

DnAB helicase is a ring-shaped hexamer of 300 kDa that is essential for replication of the bacterial chromosome. The *dnaB* gene from *Thermus aquaticus* was isolated and cloned, and its gene product was expressed and purified to homogeneity. A helicase assay was developed, and optimal conditions for *T. aquaticus* DnAB activity were determined using a forked duplex DNA substrate. The activity required a hydrolyzable nucleoside triphosphate and both 5′- and 3′-single-stranded DNA tail regions. Under conditions of single enzymatic turnover, the lengths of the 5′- and 3′-single-stranded regions were varied, and 6–10 nucleotides of the 5′-single-stranded tail and 21–30 nucleotides of the 3′-single-stranded tail markedly stimulated the unwinding rate. These data suggest that DnAB from *T. aquaticus* interacts with both DNA single-stranded tails during unwinding and that a greater portion of the 3′-tail is in contact with the protein. Two models are consistent with these data. In one model, the 5′-single-stranded region passes through the central hole of the DnAB ring, and the 3′-tail makes extensive contact with the outside of the protein. In the other model, the 3′-single-stranded region passes through the DnAB ring, and the outside of the protein contacts the 5′-tail.

DnAB, a ring-shaped hexamer of ~300 kDa (1–3) is essential for replication of the bacterial chromosome (4). It is also involved in the replication of bacterial phage (5, 6) and plasmid DNA (7). It has been proposed that the protein unwinds double-stranded DNA at a replication fork, providing single-stranded DNA templates for DnaG primase and DNA polymerase III holoenzyme (8). The protein exhibits helicase activity *in vitro*, catalyzing the conversion of double-stranded DNA to single-stranded DNA with concomitant hydrolysis of ATP (9, 10).

DnAB is homologous to two other characterized hexameric helicases involved in DNA replication, the gene 4 protein from *T. aquaticus* (9, 10). We are interested in studying the mechanism of DnAB-catalyzed unwinding by determining the crystal structure of DnAB in complex with forked duplex DNA. Proteins from thermophilic organisms are often easier to purify and may crystallize more readily than their mesophilic counterparts. Therefore, we isolated and cloned the gene for *Thermus aquaticus dnaB*, overexpressed its gene product, and purified the protein to homogeneity.

To design DNA substrates for cocrystallization with DnAB and to begin establishing its mechanism, we need to know what single-stranded regions of DNA are contacting the protein during unwinding. To address this issue, we developed a helicase assay using a forked duplex DNA substrate. We then varied the lengths of the single-stranded tail regions and found that the first 6–10 nucleotides of the 5′-tail and the first 21–30 nucleotides of the 3′-tail emanating from the duplex region markedly stimulated the unwinding rate. Thus, these data suggest that the 3′-tail makes more extensive contacts with *T. aquaticus* DnAB than the 5′-tail. Two models may account for these data. In one, the 5′-single-stranded tail passes through the central hole of the protein helicase, whereas the 3′-tail makes more extensive contact with the outside of the protein toroid. In the second model, the 3′-tail passes through the central hole, and the 5′-tail makes less extensive contact with the outside of the protein ring.

These two alternative models would position the protein ring around the lagging or leading strands of the DNA, respectively, during replication.

**EXPERIMENTAL PROCEDURES**

*Isolation and Cloning of the dnaB Gene from *T. aquaticus***

Genomic DNA from *T. aquaticus* YT-1 strain was prepared as described previously (22). To obtain the initial PCR product, 50-μl reactions were set up with 50 ng of genomic DNA, 20 μM each primer, 3.3 mM dGTP and dCTP, 1.7 mM dATP and dTTP, 1× Vent buffer (New England Biolabs), and 1 μl of Deep Vent DNA polymerase (New England Biolabs). The forward primer was GG(GC)TC/(GC)ATC(GC)GAGCCCCGAGCG(GC)(GA)(CT), which was designed to include the amino acid region corresponding to 406 to 413 of DnAB from *E. coli*. The reverse primer was 5′-(GC)GG(GC)(CGTT)(GC)(CGTG)CT(CT)T3′, which was designed to be complementary to residues 439–445. For the PCR, the

---

* This work was supported in part by grants from the National Institutes of Health. 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.

**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank*™/EBI Data Bank with accession number(s) AF100420.**

‡ Howard Hughes Medical Institute Predoctoral Fellow.

¶ Howard Hughes Medical Institute Predoctoral Fellow.

§ Howard Hughes Medical Institute Predoctoral Fellow.

**To whom correspondence should be addressed: Dept. of Molecular Biophysics and Biochemistry, Yale University, 266 Whitney Ave., New Haven, CT 06520-8114. Tel.: 203-432-5617; Fax: 203-432-3282.

1 The abbreviations used are: PCR, polymerase chain reaction; GTPγS, guanosine-5′-γ-thio-triphosphate; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; ATPγS, adenosine-5′-γ-thio-triphosphate; Mes, 2-(N-morpholino)ethanesulfonic acid; DTT, dithiothreitol; Bicine, N,N-bis(2-hydroxyethyl)glycine.
melting conditions were 1 min at 95 °C, the annealing conditions were 1 min at a temperature that was decreased stepwise from 60 to 45 °C in 0.5°C increments (31 cycles), and the extension conditions were 2.5 min at 72 °C.

To isolate regions 5’ and 3’ to the initial product, inverse PCR was performed. Templates for inverse PCR were prepared by digesting T. aquaticus genomic DNA with various restriction enzymes. The restriction enzymes were chosen to yield products of average size between 500 and 2000 base pairs. The average size product was estimated by determining the frequency of a particular restriction site within all T. aquaticus genomic DNA sequence that had been deposited in the data base. After the restriction enzyme had been heat inactivated, the DNA was religated at a low (2 mM) DNA concentration to favor unimolecular reactions, thus yielding circular fragments of genomic DNA. Ligation reactions were performed with T4 DNA ligase (New England Biolabs) according to the instructions of the manufacturer. Inverse PCR was performed using primers directed away from each other and against regions of dnaB that had been sequenced. The PCRs were set up as above except that the annealing temperature was decreased in 0.3 °C increments from 70 °C to 60 °C (35 cycles). Pfu DNA polymerase (Stratagene) instead of Deep Vent DNA Polymerase was used in some of these inverse PCRs.

To isolate the entire gene, rTth DNA polymerase, XL (Perkin-Elmer) was used. The forward primer contained the N terminus of the gene, and the reverse primer was complementary to a region 3’ to the C-terminus. The PCR was performed as above with a 5-min extension time. The full-length gene was cloned into a pET-22b(+) vector (Novagen) using NdeI and SacI restriction sites.

Expression and Purification of the dnaB Gene Product from T. aquaticus

Expression, Lysis, and Heat Treatment—The pET22b(+) vector containing the dnaB gene was transformed into BL21 cells. The transformed cells were grown in 10 liters of LB medium containing 50 μg/ml ampicillin in a stirred, aerated fermentor at 37 °C. When the cells reached an A600 of 0.6, the temperature was decreased to 30 °C, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 100 μM to induce gene expression. Three hours later, 34 g of cells were harvested by centrifugation. From this point, all manipulations were carried out at 4 °C unless otherwise stated. The 34 g of cells were resuspended in 200 ml of Buffer A, which contained 10% sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 5 mM DTT. The cells were then lysed with a French press. MgCl2 was then added to a final concentration of 5 mM. The lysate was then heated at 65 °C for 20 min followed by chilling on ice for 20 min. The cells were then spun at 38,000 rpm in a T45 rotor (Beckman) for 60 min. The supernatant was filtered with a 0.22 μm low protein binding filter (Fraction I).

A Q-Sepharose column (Amersham Pharmacia Biotech) was preequilibrated with Buffer B, which contained 10% glycerol, 50 mM Heps, pH 7.0, 50 mM NaCl, 5 mM MgCl2, and 5 mM DTT. Fraction I was loaded onto the column and the column was then washed with 5 column volumes of Buffer B. The protein was eluted with a linear gradient of Buffer B to Buffer C containing an additional 450 mM NaCl. DnaB eluted at 300–945 mM NaCl (Fraction II).

Heparin-Sepharose Column—Fraction II was dialyzed against Buffer C, which contained 10% glycerol, 20 mM Heps, pH 7.0, 50 mM NaCl, 5 mM MgCl2, 5 mM DTT, and 0.02% sodium azide. After dialysis, the sample was loaded onto a HiTrap heparin-Sepharose column (Amerham Pharmacia Biotech) that was preequilibrated with Buffer C. The column was then washed with 5 column volumes of Buffer C followed by elution with a linear gradient of Buffer C to Buffer D containing an additional 450 mM NaCl. DnaB eluted between 200 and 250 mM NaCl (Fraction III).

Concentration—Fraction III was dialyzed against Buffer D, which contained 10% glycerol, 20 mM Heps, pH 7.0, 100 mM NaCl, 5 mM MgCl2, 5 mM DTT, and 0.02% sodium azide. The sample was then concentrated with an Amicon Ultrafiltration device followed by an Amicon Centricon device. The concentration of protein was 11.1 mg/ml at this point. The final yield was 112 mg of purified protein from a 10-liter cell growth. 80% glycerol was added to the sample to achieve a final concentration of 50% glycerol. The sample was then stored at −20 °C until further use.

Protein Analysis

A reducing, 10–20% polyacrylamide gel was run in SDS using the Laemmli buffer system (23). The gel was run at a constant 175 V for 45 min and then stained with Coo massie Blue as described (24). Molecular weight marker standards were from Amersham Pharmacia Biotech (catalog no. 17-0446-01). Accurate protein concentration and amino acid content were determined by performing amino acid analysis on triplicate samples of purified protein. This analysis was carried out by the Howard Hughes Medical Institute Biopolymer/W. M. Keck Biotechnology Resource Laboratory at Yale University (Keck Facility) on a Beckman model 6300 ion exchange instrument following acid hydrolysis.

DNA Analysis

All DNA sequencing was performed by the Keck Facility using Applied Biosystems 377 DNA sequencers. The sequence reactions utilize fluorescently labeled dideoxynucleotides (dRhodamine Terminators) and Taq FS DNA polymerase in a thermal cycling protocol.

Helicase Assay

Substrate Preparation—All DNA oligomers were synthesized by the Keck Facility and gel-purified. The 3’-tailed oligomer was labeled at the 5’-end with T4 polynucleotide kinase (New England Biolabs). The kinase was denatured by heating to 65 °C for 20 min. The unincorporated nucleotides were removed with a G-50 Sephadex spin column. This labeled strand was mixed with a 1:1.2 ratio with the unlabeled, complementary strand. Identical helicase reaction rates were obtained when this ratio was 1:1 (data not shown), and the excess unlabeled strand was added to enhance annealing. Two equivalent unlabeled strands of the same 1:2 ratio were added to the labeled mixture in a 5-fold excess. The unlabeled mixture was added in excess to ensure that the concentration of substrate was accurate. The strands were then annealed by heating to 95 °C for 5 min followed by slow cooling to room temperature. The strands were then preannequenced at 55 °C for 3 h. This step ensured that the percentage of duplex remained constant throughout the assay in the absence of enzyme.

Assay Conditions—Standard conditions for the helicase assay were as follows: 500 μM T. aquaticus DnaB helicase, 5 μM GTP, 1 μM DNA substrate, 10% glycerol, 25 mM sodium Bicine, pH 9.0, 50 mM potassium glutamate, 10 mM NaCl, 10 mM MgCl2, 1.5 mM DTT, 0.1 mM γ-32P-labeled bovine serum albumin, and 55 °C. The reagents in the assay were added in the following order on ice: buffer, DNA substrate, enzyme, and finally GTP. After incubation at 55 °C for the time indicated in the text, the reactions were quenched by placing on ice and adding stop buffer to achieve a final concentration of 1% SDS, 40 mM EDTA, 20% glycerol, and 0.1% xylene cyanol. The samples were then snap frozen in liquid nitrogen and stored at −20 °C until they were ready for final analysis by native polyacrylamide gel electrophoresis.

Single strand product was separated from duplex substrate by running the samples through a 12% polyacrylamide gel (19:1 acrylamide: bis) in 1× TBE (90 mM Tris-Borate, 2 mM EDTA) at 5 W at room temperature for 1–2 h. The variation in time depended upon the lengths of the DNA species in the assay. Quantitation of Product—After the gel was run, it was dried at 50 °C for 40 min. The gel was then exposed to a Bio-Imaging Plate (Fuji Photo Film Co.). Band intensities were quantified and background counts subtracted using the MacBAS software package. The percentage of single strand was typically ~0–10% in an untreated sample and ~95–100% in a heat-denatured sample. To normalize for the slight variability in these values, the percentage of product was calculated using the following equation,

\[ \% \text{Product} = \frac{\% S}{\% S + \% D} \]  
(Eq. 1)

where \%S is the percentage of single strand in the sample of interest, \%D is the percentage of single strand in the untreated duplex lane, and \%S is the percentage of single strand in the heat-denatured lane.

RESULTS

Isolation, Cloning, Expression, and Purification of DnaB from T. aquaticus—The dnaB gene from T. aquaticus isolated and cloned using the technique of PCR. The complete amino acid sequences of DnaB from five other bacterial species had been deposited in the database at the time of cloning. Based on these sequences and the codon usage frequency of T. aquaticus, degenerate oligonucleotide primers for PCR were designed (25, 26). Using genomic DNA as a template, a 120-base pair DNA fragment was initially isolated and sequenced. This fragment encoded an amino acid sequence that is highly similar to

T. aquaticus DnaB Helicase Activity
DnaB from other bacterial species within the region between the primers. The technique of inverse PCR was then used to isolate regions 5′ and 3′ to the original sequence (27). The N terminus of the gene was identified by a Shine-Dalgarno sequence (GGTAGG) situated 10 base pairs upstream to an ATG initiation codon. This 5′-region was amplified and sequenced in triplicate because it was used as a primer for subsequent PCR gene amplification. The entire gene was PCR-amplified in triplicate using one primer containing the 5′-initiation site and a second primer complementary to a region 3′ to the C terminus. These three products were then cloned and sequenced. Because amplification and sequencing were performed in independent, triplicate experiments the gene sequence was unambiguously confirmed.

The predicted amino acid sequence of the dnaB gene product yields a protein of 49 kDa per monomer. The GC content of the gene is 67%, typical of genes from this species. The protein has 48% amino acid identity and 69% amino acid similarity compared with DnaB from E. coli. Regions within this gene that are highly conserved among other species (12) are well conserved for *T. aquaticus* DnaB. The protein has 1 cysteine and 14 methionines, which may be useful in future crystallography work.

The *T. aquaticus* dnaB gene was then cloned into a T7 expression system (28). Induction and purification of the DnaB gene product were carried out as described under “Experimental Procedures.” The final protein sample was ~95% pure as judged by Coomassie Blue staining of a reducing SDS-polyacrylamide gel electrophoresis gel (not shown). The protein ran according to its predicted molecular weight on this gel. Once purified protein was obtained, amino acid analysis was performed to confirm the correct identity of the product and to determine an accurate extinction coefficient, which was calculated to be ε280 = 0.46 liter g⁻¹ cm⁻¹.

**Development of a Helicase Assay**—To determine what 5′- and 3′-tail lengths of a forked duplex DNA substrate are bound by *T. aquaticus* DnaB during unwinding, we developed a helicase assay. In this assay, the rates of unwinding under conditions of single enzyme turnover are measured for a series of forked duplex DNA substrates with various 5′- and 3′-single-stranded tail lengths. As the tail is shortened beyond a length that is critical for binding and/or unwinding, a marked decrease in helicase rate should be observed.

The standard substrate used in this helicase reaction had a 22-base pair duplex region and contained a 30-nucleotide deoxythymidylicate (d(pT)30) in each single-stranded tail region (Fig. 1). The duplex region had a melting temperature of ~65 °C, and the poly(T) base composition of each tail ensured that there was no interaction between the tails. The strand with the 3′-tail was labeled at the 5′-end with ³²P, and the conversion of this strand from duplex to single strand was monitored with native gel electrophoresis.

Conditions for the helicase assay were optimized to achieve a maximum rate of product formation. These conditions are described under “Experimental Procedures.” Briefly, 500 nM enzyyme, 1 nM DNA substrate, and 5 mM GTP were incubated at pH 9.0 at 55 °C for various times. A low substrate concentration was used to slow the rate of product reannealing. Furthermore, enzyme concentration was in vast excess compared with substrate concentration, ensuring that the kinetics observed were for single-enzyme turnover. Multiple-turnover kinetic data can be dominated by Kₐₜ rates and may be inadequate to assess productive binding. A time course for this helicase assay was then performed (Fig. 2A, lanes 1–13, and Fig. 2B). Nearly 50% of the substrate was converted to product in 2 min in the presence of 5 mM GTP, but there was no observable unwinding without added GTP.

Although protein-dependent conversion of double-stranded DNA to single-stranded DNA is the hallmark of helicase activity, if the substrate and product are in rapid exchange at equilibrium, a reagent with single-stranded binding activity can sequester the product and drive the equilibrium toward product formation. This nonenzymatic activity is seen for single-stranded binding proteins such as *E. coli* single-stranded binding protein and T4 gp32 (29). To determine whether this could occur under these assay conditions, a 22-nucleotide single-stranded DNA oligomer that was complementary to the duplex region of the labeled strand was added to the reaction in ≥20-fold excess. This “trap” DNA oligomer contained no 5′-single-stranded tail. If melting occurs, this excess DNA should bind to the released labeled single strand and inhibit the reverse reaction. Because this excess unlabeled strand contained no tail region, its duplex with labeled strand could be electrophoretically separated from substrate. After 2 h, there was only a ~6% decrease in substrate concentration, suggesting that the substrate and product were not in rapid exchange relative to the time course of the reaction (Fig. 2A, lanes 14–19, and Fig. 2C).

**Reaction Requirements for Helicase Activity**—Enzymatic unwinding is characterized by coupled hydrolysis of a nucleoside triphosphate. To determine whether the helicase activity mediated by *T. aquaticus* DnaB was dependent upon nucleoside triphosphate hydrolysis, the reaction was performed in the presence of protein and various nucleotide analogs (Fig. 3A). After a 30-min incubation, nearly all of the substrate was converted to product in the presence of 5 mM ATP or GTP. By contrast, incubation with the same concentration of the nucleoside diphosphate GDP yielded no product. Similarly, incubation with the slowly hydrolyzable analogs GTPγS, AMP-PNP, and AMP-PCP yielded only 9, 4, and 3% product, respectively, after 30 min. These data suggest that hydrolysis of a nucleoside triphosphate is required for helicase activity by *T. aquaticus* DnaB. Incubation with either ADP or ATPγS resulted in accumulation of radiolabel in the well of the gel (“well shift”) that was difficult to reverse. We therefore cannot assess the enzymatic conversion to product in the presence of these analogs.

Other nucleoside triphosphates could stimulate the helicase activity of DnaB. As shown in Fig. 3B, incorporation of any of the ribonucleoside triphosphates resulted in nearly complete conversion to product in 30 min, whereas the deoxyribonucleoside triphosphates...
were generally less effective.

The protein concentration dependence for the reaction, expressed in hexamers, is shown in Fig. 4A. DnaB at a concentration of 5 nM resulted in nearly 50% product in 30 min, and 50 nM DnaB resulted in nearly 90% conversion to product. These concentrations were in excess of substrate, which was at 1 nM concentration. The large excess of protein required here for activity is atypical for an enzymatic reaction. However, this relationship is typical for the hexameric helicases (10, 13, 16). We also noted a log-linear relationship between protein concentration and activity. Again, this is not typical of most enzymatic reactions, but it has been reported for the T4 gene 41 helicase (13). The unusual concentration dependence for hexameric helicase activity may be explained if the enzyme dissociates at low protein concentrations.

There was a steep pH dependence for activity, with the protein was far more active under alkaline conditions (Fig. 4B). Greater than 90% conversion to product was observed at pH 8 or 9. Because the activity increase was largest in the pH range 5–8, it is more likely that some group(s) on the protein was being titrated as opposed to the DNA because DNA has no group with a pKₐ in this pH range.

The salt dependence of the reaction was also determined. The addition of 50 mM potassium glutamate resulted in a slight enhancement of activity compared with 0 mM potassium glutamate (Fig. 4C). Each reaction contained 10 mM NaCl from the protein storage buffer, as well as 25 mM sodium Bicine and 10 mM MgCl₂. Higher concentrations of potassium glutamate were inhibitory in a concentration-dependent manner (Fig. 4C). These data are consistent with salt-mediated disruption of a critical ionic interaction, possibly between the protein and the DNA. Replacing potassium glutamate with sodium glutamate, sodium chloride, or potassium chloride resulted in very little change in the percentage of product formed (not shown).

**Helicase Rates Using Single-tailed Substrates and Estimation of Reannealing Rates**—To examine the role of the 5’- and 3’-single-stranded tail regions in the reaction, the assay was performed with a substrate containing a d(pT)₃₀-5’-tail and no 3’-tail or with a substrate containing a d(pT)₃₀-5’-tail and no 3’-tail. In either case, there was no conversion to product within a 2-h time course, indicating that both tails are required for activity (not shown). These data further support the claim that DnaB is acting as a helicase and not a single-stranded binding protein because it is unlikely that product binding is two-tail-dependent.

A helicase assay is complicated by the fact that the single-stranded products can reanneal to form substrate. Thus, it is important to measure the rate of product reannealing. A substrate containing a d(pT)₃₀-5’-tail and no 3’-tail was heat-denatured and then allowed to reanneal in the absence and presence of protein under the conditions used in these assays. This substrate was chosen because DnaB could not unwind it. Without protein, the half-time for reannealing was ~30 min (Fig. 5). However, the half-time for reannealing increased to ~120 min in the presence of DnaB, suggesting that the enzyme inhibits reannealing. This inhibition may be caused by transient protein interaction with the single-stranded products (30, 31). Because the results presented here are based upon the first 30 min of the reaction, product reannealing will not substantially alter the reaction kinetics presented. However, the percentage of product reported will slightly underestimate the
total product produced, particularly at 30-min time points.

Effect of 5’- and 3’-Tail Length on Helicase Rate—The length of the 5’-tail was then varied stepwise from d(pT)30 to d(pT)0, whereas the 3’-tail length remained constant at d(pT)10. There was a very slight decrease in helicase rate as the length of the 5’-tail was decreased stepwise from d(pT)30 to d(pT)10 (Fig. 6A).

However, there was a substantial decrease as the 5’-tail length was shortened from d(pT)10 to d(pT)5 and from d(pT)5 to d(pT)0. Thus, from the duplex region, the first 6–10 deoxythymidylicates of the 5’-tail markedly stimulate unwinding, but d(pT)s 11–30 have little effect. These data suggest that only the first 6–10 d(pT)s of the 5’-tail productively interact with DnaB during this helicase reaction.

The length of the 3’-tail was then varied stepwise from d(pT)30 to d(pT)0 while the 5’-tail length remained constant at d(pT)0. There was a measurable decrease in helicase rate as the 3’-tail length decreased stepwise from d(pT)30 to d(pT)0, suggesting that most of this region stimulated the unwinding rate and therefore may contact the protein during the reaction (Fig. 6B).

A substrate with a d(pT)10-5’-tail and a d(pT)10-3’-tail was converted to product at the same rate as one containing a d(pT)30-5’-tail and a d(pT)30-3’-tail (Fig. 6C). These data suggest that the stimulatory effect of d(pT)s 11–30 of the 3’-tail is independent of d(pT)s 11–30 of the 5’-tail. Likewise, the inability of d(pT)s 11–30 of the 5’-tail to stimulate the unwinding rate is independent of d(pT)s 11–30 of the 3’-tail.

The above results suggest that DnaB from T. aquaticus contacts the first 6–10 nucleotides of the 5’-tail and the first 21–30 nucleotides of the 3’-tail as the helicase unwinds duplex DNA. Thus, DnaB may have more extensive contact with the 3’-tail than the 5’-tail during unwinding. This possibility is best exhibited by the substantially increased unwinding rate of the forked duplex containing a d(pT)10-5’-tail and a d(pT)30-3’-tail compared with one containing a d(pT)30-5’-tail and a d(pT)10-3’-tail (Fig. 6D).

The unwinding of these substrates was also compared in reactions containing ATP instead of GTP because ATP is likely to be the predominant cellular cofactor. When these samples were analyzed by native gel electrophoresis, a substantial well shift was seen under several conditions (Fig. 7A). Therefore, all of the samples were incubated with 20 mM of the single-stranded oligomer d(pT)30 for 1 h at room temperature and reanalyzed. Incubation with this oligomer effectively removed the well shift but did not alter the single strand percentage in lanes with no well shift (Fig. 7B). Again, nucleotides 11–30 of the 3’-tail markedly stimulated the unwinding rate, but nucleotides 11–30 of the 5’-tail had little effect. Thus, a similar asymmetric tail length dependence was found for reactions containing either GTP or ATP as a cofactor (Fig. 7, B and C).

Finally, the deoxythymidylicates in either tail were replaced with deoxyadenylates to determine whether there was a difference between pyrimidine- and purine-containing tails. A similar asymmetric tail length dependence was observed (Fig. 8). Thus, DnaB from T. aquaticus may productively interact with a greater region of the 3’-single-stranded tail region as compared with the 5’-tail region. This effect is relatively insensi-

![Fig. 3. Nucleotide requirement for the helicase reaction. The standard substrate shown in Fig. 1 was used in these 30-min reactions. A, native gel analysis of the helicase reaction performed in the presence of 5 mM of various nucleotides and nucleotide analogs. B, the helicase reaction was performed in the presence of 5 mM of various nucleoside triphosphates. Analyses were performed as described under “Experimental Procedures.”](image)

![Fig. 4. Reagent requirements for the helicase reaction. The standard substrate shown in Fig. 1 was used in these reactions. A, the percentage of product as a function of hexamer concentration is shown for 30-min incubations. B, the percentage of product as a function of pH is shown for 30-min incubations. The buffers used were sodium acetate (5.0), sodium Mes (6.0), sodium Hepes (7.0 and 8.0), or sodium Bicine (9.0). C, the percentage of product as a function of potassium glutamate concentration is shown for 8-min (open circles) and 30-min (filled circles) incubations.](image)
tive to the nucleoside triphosphate or the single-stranded tail sequence present in the reaction.

**DISCUSSION**

**Novel Tail Length Dependence Observed for DnaB Helicase Activity**—The dnaB gene from *T. aquaticus* was isolated and cloned, and its protein product was overexpressed and purified to homogeneity. The gene product shares 48% identity and 69% similarity compared with its *E. coli* homologue. A helicase assay for this protein was developed using a forked duplex DNA substrate. The single-stranded tail length dependence for unwinding was determined under conditions of single-enzyme turnover to determine the DNA regions that productively interact with DnaB helicase. These data will be useful in designing substrates for cocrystallization studies.

From the duplex region, the first 21–30 nucleotides of the 3′-single-stranded tail and the first 6–10 nucleotides of the 5′-single-stranded tail substantially stimulate the unwinding rate of *T. aquaticus* DnaB. Nucleotides 11–30 of the 5′-tail exhibit no effect or slightly stimulate activity, depending upon the base composition of the tails and the nucleotide cofactor used in the experiment. Thus, a larger portion of the 3′-tail stimulates helicase activity compared with the 5′-tail, a result that has not previously been observed for proteins of this family.

**Polarity of DnaB Family Helicases**—Helicases can exhibit either 5′ to 3′ or 3′ to 5′ polarity with respect to the DNA strand that they are bound to. Gene 41 protein from T4 phage and gene 4 protein from T7 phage have been shown to exhibit 5′ to 3′ polarity (13, 14, 16). There is both direct (10) and inferential (32, 33) evidence that *E. coli* DnaB unwinds DNA with 5′ to 3′ polarity. Most helicases require just one single-stranded tail region for duplex unwinding. However, for members of the DnaB protein family, both single-stranded tail regions are required (10, 14, 16). Thus, polarity is more difficult to assess for these proteins because the protein is contacting both tails, and under some conditions, *E. coli* DnaB has been shown to exhibit 3′ to 5′ polarity (10). Furthermore, because the DNA tail regions used in these polarity experiments are quite long, the specific DNA regions that interact with the helicase cannot be adequately addressed with these studies alone.

**Helicase Assay Data Are Complementary to Dissociation Constant and Electron Microscopy Data**—Equilibrium dissociation constant data for *E. coli* DnaB bound to DNA have been previously obtained and are complementary to those of our unwinding assay. Fluorescence data show that the site size for *E. coli* DnaB binding to single-stranded DNA is 20 ± 3 nucleotides (34). Fluorescence experiments have also been performed to determine how *E. coli* DnaB binds forked duplex DNA in the presence of the nonhydrolyzable ATP analog AMP-PNP (35, 36). These studies conclude that DnaB binds to either the 5′- or 3′-single-stranded tail regions, but the protein does not bind to both tails simultaneously (36). There was a 6–20-fold lower *Kd* for 5′-tail binding compared with 3′-tail binding (35, 36). It was also concluded that DnaB was in two opposite orientations with respect to the duplex region of the DNA depending on the

---

**Fig. 5.** Estimation of single-stranded product reannealing rates. A substrate identical to that shown in Fig. 1 except with no 3′-single-stranded tail region was heated to 95 °C for 5 min. The single-stranded products were then allowed to reanneal under the standard experimental reaction conditions in the absence (open circles) or presence (filled circles) of 500 nM *T. aquaticus* DnaB.

**Fig. 6.** Effect of 5′- or 3′-tail length on the helicase rate. A and B, the length of the 5′-single-stranded tail region (A) or the 3′-single-stranded tail region (B) of the standard substrate shown in Fig. 1 was decreased in stepwise increments (d(pT)10, filled circles; d(pT)15, open circles; d(pT)20, filled squares; d(pT)25, open squares; d(pT)30, filled triangles; d(pT)35, open triangles). The other single-stranded tail region was a constant d(pT)30. C, helicase rates for substrates containing d(pT)30-5′ and d(pT)30-3′-tails (filled circles), d(pT)10-5′ and d(pT)30-3′-tails (filled triangles), d(pT)15-5′ and d(pT)30-3′-tails (open triangles), or d(pT)20-5′ and d(pT)30-3′-tails (x). D, native gel analysis of the helicase reaction performed using a substrate containing d(pT)10-5′ and d(pT)20-3′-tails (lanes 1–10, 10-T-5′_30-T-3′), or d(pT)15-5′ and d(pT)25-3′-tails (lanes 11–20, 30-T-5′_10-T-3′).
It is difficult to directly relate these binding constant data to those of our helicase assay for several reasons. First, the two-tailed dependence for DnaB unwinding observed here and elsewhere (10) was not seen in these steady-state binding experiments. Second, it is not known with certainty which protein orientation is directed toward unwinding the duplex region in these binding assays. Finally AMP-PNP may render DnaB in a different conformational state compared with ATP or GTP. Electron microscopy studies have been performed with *E. coli* DnaB and with gene 4 from T7. For both proteins, the 3-dimensional shape of the hexamer is that of a ring-shaped structure with an internal cavity diameter of ~25–40 Å, which is large enough to accommodate single-stranded DNA (2, 3, 20, 21). These proteins are highly processive, and they may remain bound to the DNA throughout replication (18, 19). High processivity may be conferred by having one of the DNA single strands pass through the hexamer central cavity. In the case of the T7 gene 4 protein, single-stranded DNA is passing through the central hole of the protein ring in the electron microscopy image (20, 21). Recently, fluorescence data have indicated that a DNA single strand passes through the inner channel of *E. coli* DnaB (37).
Two Possible Models for DnaB Interaction with a Replication Fork—Two likely models describe *T. aquaticus* DnaB interaction with a replication fork. In the first model, the 5′-single stranded tail region passes through the central hole of the protein hexamer, whereas the 3′-tail contacts the outside of the protein (Fig. 9A). This model positions the protein around the lagging strand during DNA replication. From the replication fork, only the first 6–10 nucleotides of the strand that passes through the interior of the protein would productively interact with the helicase during unwinding. Moreover, the 21–30 nucleotides of the strand that does not pass through the protein, the leading strand, may contact the outside of the protein. This model is consistent with the 5′ to 3′ polarity that has been demonstrated for *E. coli* DnaB and other members of this helicase family. However, the length of the 5′-single-stranded tail region of DNA that productively interacts with the interior of DnaB in this model, 6–10 nucleotides, is somewhat less than the single-stranded DNA length bound by *E. coli* DnaB, 20 ± 3 nucleotides (34). It is possible that the region of 5′-single-stranded tail DNA located ~11–20 nucleotides from the duplex passes through the central cavity of DnaB but does not productively contact the protein as measured by this unwinding assay.

A second model is also possible. In this case, the 3′-single stranded tail would pass through the central hole of the protein ring, and the outside of the protein would then contact the 5′-single-stranded region (Fig. 9B). This model would position the hexamer around the leading strand of the replication fork. For *T. aquaticus* DnaB, the 21–30 nucleotides of the strand that passes through the protein, the 3′-tail, would now contact the helicase. The outside of the hexamer would contact 6–10 nucleotides of the 5′-tail, which corresponds to the lagging strand.

In this second model, the length of leading-strand DNA passing through and contacting the *T. aquaticus* DnaB protein, 21–30 nucleotides, is consistent with the single-stranded DNA length bound by *E. coli* DnaB, 20 ± 3 nucleotides (34). However, this model may contradict previously proposed models of 5′ to 3′ movement polarity for members of this helicase family. This model also contradicts the report based upon fluorescence data that the 5′ single-stranded tail region of a forked DNA substrate passes through the inner channel of *E. coli* DnaB (37). This apparent discrepancy can be explained if the orientation of the DnaB protein is reversed in these fluorescence experiments, such that the protein is positioned to move away from the duplex region. The ability of DnaB to encircle the 3′-single stranded tail region of a forked DNA substrate has not yet been assessed.

Because all polymerases have 5′ to 3′ polarity of synthesis, primase must use the lagging strand as a template to initiate each cycle of Okazaki fragment synthesis. For T7 phage, the gene 4 protein is responsible for both helicase and primase activities. For *E. coli*, DnaB helicase binds to DnaG primase with weak affinity (38). Thus, in either system, the replication fork helicase interacts with the primase. In the first model presented above, the helicase is encircling the template strand for primase, whereas in the second model, the helicase is surrounding the opposite strand (Fig. 9). Thus, the interaction between primase and helicase will be predominantly same-strand in the first model and cross-strand in the second model. Either model is possible, because it has recently been shown that the T7 gene 4 protein can use either the strand that it is bound to or a foreign strand as a template for priming (39).

While this work was in progress, two studies performing similar experiments with the gene 4 protein from T7 phage were reported (15, 17). In these studies, the tail length dependence on unwinding rate is different from that presented here for DnaB. These studies show that ~35 nucleotides of the 5′-tail stimulate unwinding, whereas only ~10–15 nucleotides of the 3′-tail stimulate unwinding. Thus, the tail length asymmetry observed for the gene 4 protein is nearly the reverse of that shown here for *T. aquaticus* DnaB. It is likely that these two proteins unwind duplex DNA with a similar mechanism because they are homologous. However, we are led to wonder whether it is possible that the two related helicases encircle different strands but still retain the same overall mechanism, which would result in the 5′-tail passing through the center of T7 gene 4 protein, as in the first model presented above, and the 3′-tail passing through the center of *T. aquaticus* DnaB, as in the second model presented above. Alternatively, each of these proteins may surround the single-stranded tail at the replication fork, but the specific DNA contact may be different. Future structural studies will help determine which of these hypotheses is correct.

Acknowledgments—We thank Drs. Yousif Shamoo and Satwik Kamtekar for comments on the manuscript. We also thank Dr. Anna Lee for help in preparing Fig. 9.

Note Added in Proof—Since submitting this manuscript for review, we have performed unwinding assays using forked duplex DNA substrates that are composed of oligonucleotides of mixed polarity. We found that DnaB from *T. aquaticus* unwinds forked duplex DNA containing two 5′ single-stranded regions at an equal rate compared to the standard substrate. By contrast, the enzyme could not unwind forked duplex DNA containing two 3′ single-stranded tails. These new observations suggest that the generally accepted model shown in Fig. 9A is more likely than the alternative model illustrated in Fig. 9B.

REFERENCES
1. Bojalskiwski, W., Klenowska, M. M., and Jezewska, M. J. (1994) *J. Biol. Chem.* 269, 31350–31358.
2. Martin, M. C. S., Stamford, N. P. J., Dammerova, N., and Dixon, N. E. (1995) *J. Struct. Biol.* 114, 167–176.
3. Yu, X., Jezewska, M. J., Bojalskiwski, W., and Egelman, R. H. (1996) *J. Mol. Biol.* 259, 7–14.
4. Wechsler, J. A., and Gross, J. D. (1971) *Mol. Gen. Genet.* 113, 273–284.
5. Arai, K.-I., and Kornberg, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 4308–4312.
6. Arai, K.-I., and Kornberg, A. (1981) *J. Biol. Chem.* 256, 5267–5272.
7. Kaguni, J. M., and Kornberg, A. (1984) *Cell* 38, 183–190.
8. Baker, T. A., Funnell, B. E., and Kornberg, A. (1987) *J. Biol. Chem.* 262, 6877–6885.
9. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A. (1986) *Cell* 45, 53–64.
10. LeBowitz, J. H., and McMacken, R. (1986) J. Biol. Chem. 261, 4738–4748
11. Gorbalenya, A. E., and Koonin, E. V. (1993) Curr. Opin. Struct. Biol. 3, 419–429
12. Ilyina, T. V., Gorbulenyua, A. E., and Koonin, E. V. (1992) J. Mol. Evol. 34, 351–357
13. Venkatesan, M., Silver, L. L., and Nossal, N. G. (1982) J. Biol. Chem. 257, 12426–12434
14. Richardson, R. W., and Nossal, N. G. (1989) J. Biol. Chem. 264, 4725–4731
15. Matson, S. W., Taber, S., and Richardson, C. C. (1983) J. Biol. Chem. 258, 14017–14024
16. Ahnert, P., and Patel, S. S. (1997) J. Biol. Chem. 272, 32267–32273
17. Mok, M., and Marians, K. J. (1987) J. Biol. Chem. 262, 16644–16654
18. Wu, C. A., Zechner, E. L., and Marians, K. J. (1992) J. Biol. Chem. 267, 4030–4044
19. Yu, X., Hingorani, M. M., Patel, S. S., and Egelman, E. H. (1996) Nature Struct. Biol. 3, 740–746
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Section 18.55, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY