Asymmetric Interactions of Hexameric Bacteriophage T7 DNA Helicase with the 5′- and 3′-Tails of the Forked DNA Substrate*

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Bacteriophage T7 DNA helicase requires two noncomplementary single-stranded DNA (ssDNA) tails next to a double-stranded DNA (dsDNA) region to initiate DNA unwinding. The interactions of the helicase with the DNA were investigated using a series of forked DNAs. Our results show that the helicase interacts asymmetrically with the two tails of the forked DNA. When the helicase was preassembled on the forked DNA before the start of unwinding, a DNA with 15-nucleotide (nt) 3′-tail and 35-nt 5′-tail was unwound with optimal rates close to 60 base pairs/s at 18 °C. When the helicase was not preassembled on the DNA, a >65-nt long 5′-tail was required for maximal unwinding rates of 12 base pairs/s. We show that the helicase interacts specifically with the ssDNA region and maintains contact with both ssDNA strands during DNA unwinding, since conversion of the two ssDNA tails to dsDNA structures greatly inhibited unwinding, and the helicase was unable to unwind past a nick in the dsDNA region. These studies have provided new insights into the mechanism of DNA unwinding. We propose an exclusion model of DNA unwinding in which T7 helicase hexamer interacts mainly with the ssDNA strands during DNA unwinding, encircling the 5′-strand and excluding the 3′-strand from the hole.

DNA helicases catalyze the conversion of dsDNA to ssDNA, deriving the required energy from nucleoside triphosphate hydrolysis (1–6). Bacteriophage T7 gene 4 protein, the subject of this study, is the primary helicase required for T7 genome replication and recombination (7). T7 helicase is a ring-shaped hexameric helicase that forms oligomers in the presence of Mg(II) and dTTP or dTMP-PCP (8). The helicase binds preferentially and tightly to ssDNA and at least one DNA-binding site is within the central hole, which can accommodate a 25–30-nt long ssDNA (9). T7 helicase requires two noncomplementary ssDNA tails next to a dsDNA region, a forked DNA substrate, to initiate DNA unwinding in vitro (10). The hexamer must therefore interact with the ssDNA tails of the forked DNA, but the exact mechanism of interaction of the helicase with the forked DNA substrate is not understood.

To date, a large number of DNA helicases have been identified, and most have been shown to require ssDNA regions for initiation of DNA unwinding in vitro (11–15). The requirement for two noncomplementary DNA tails, as for T7 helicase, is observed also in two other helicases, *Escherichia coli* DnaB helicase (16) and bacteriophage T4 DNA helicase (17), both of which are hexameric helicases (18, 19). The quaternary structures of DnaB and T4 helicases are similar to that of T7 helicase (9, 19–21), and all three helicases are required for DNA replication.

In this paper, we have investigated the interactions of T7 helicase with forked DNA substrates that mimic the topology of a replication fork. Our strategy was to investigate the specificity of the helicase for the DNA substrate by synthesizing forked DNAs containing varying lengths of the 5′- and the 3′-tails. We have used DNA unwinding to assay for protein-DNA interactions, since the unwinding activity is very sensitive to the nature of the forked DNA substrate, and the rate constants provide a quantitative measure of active interactions. Such sensitive and quantitative measurements are not possible with methods such as footprinting or DNA protection, since helicases have no sequence specificity for DNA binding (22). The single turnover DNA unwinding rate constants were measured using two assays, a fluorimetric stopped-flow assay and a radiometric gel assay. The goals of the present studies were to establish optimal reaction conditions for measuring the rate of DNA unwinding, to define the optimum forked DNA substrate for T7 helicase, and through the process understand in more detail the interactions of the helicase with the 5′- and the 3′-tails. This study represents part of our ongoing effort toward understanding the mechanism of DNA unwinding, which is not understood for any helicase.

We show that under conditions where T7 helicase was preassembled on the forked DNA, maximum unwinding rates were observed with a forked DNA that contained a 5′-tail of 35 bases. Under conditions where the helicase hexamer was not allowed to bind to the DNA until the start of the reaction, maximum unwinding rates were not achieved even with a 5′-tail >65 nt long. The optimum length of the 3′-tail under both conditions was 15 bases. Both tails were required to be ssDNA, and when each tail was made into a dsDNA by annealing complementary oligodeoxynucleotide the unwinding activity was greatly inhibited. We conclude from these studies that T7 helicase interacts asymmetrically with the ssDNA region of the 5′- and 3′-strands of the duplex DNA during DNA unwinding.

**EXPERIMENTAL PROCEDURES**

**Substrates and Reagents**

Oligodeoxynucleotides modified with fluorescein (Fig. 1) were purchased from Macromolecular Resources at Colorado State University. [γ-32P]ATP was purchased from Amersham Life Science, Inc. DNA kinase from bacteriophage T4 was purchased from Life Technologies, Inc. All other reagents were purchased from Sigma.

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1 The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; nt, nucleotide; bp/s, base pairs/s; dTMP-PCP, β,γ-methylene deoxynucleoside triphosphate.
Oligodeoxynucleotides were synthesized on a Millipore Expedite™ nucleic acid synthesis system according to standard procedures. The deprotected oligodeoxynucleotides were purified using preparative denaturing polyacrylamide gel electrophoresis. The polyacrylamide gels (10–15% acrylamide) contained 5 mM urea in TBE (23) and were run at 65 °C. The band corresponding to the desired DNA product was visualized by UV shadowing, excised, and electroeluted in an Elutrap apparatus from Schleicher & Schuell. The DNAs were ethanol-purified and stored at −20 °C before use.

DNA concentrations were determined spectrophotometrically in TBE buffer (containing 8 mM urea) by taking absorbency measurements at λ = 260 nm in a Hewlett-Packard 8452A diode array spectrophotometer. The extinction coefficients for each DNA species were calculated from the DNA sequences using extinction coefficients of bases (liter · mol⁻¹ · cm⁻¹): ε₁ = 15200, ε₁₂ = 7050, ε₁₂₀ = 12010, ε₃ = 8400.

Forked DNA Substrates

Partially double-stranded oligodeoxynucleotides, mimicking the topology of a replication fork, including a double-stranded region and two single-stranded tails, were designed as shown (Fig. 1). They were generated by annealing the appropriate ssDNA species. Annealing was carried out by mixing the DNA strands (final concentrations ranged from 300 nM to 1 μM) in TBE buffer containing 50 mM NaCl. The annealing mixture was incubated at 95 °C for 3 min and allowed to slowly cool down to 4 °C. For detection and quantitation, one strand of the forked DNA was either 5'-32P-radiolabeled as described (23) or covalently linked to a fluorescein moiety. In all cases, a 2-fold excess of the unlabeled strand was added to ensure full annealing for all labeled covalently linked DNA substrates.

Enzymes

Bacteriophage T7 gene 4A protein was expressed and purified as described previously (24). 4A is a M64L mutant of the 4A protein; the Met-64 is the initiation codon for the 4B protein. By mutating Met-64 to Leu, we were able to express 4A alone by preventing synthesis of 4B (25). The concentrations of purified proteins were determined both from absorbency measurements (ε₂₆₀ = 76,100 M⁻¹ · cm⁻¹) and by the Bradford method (26) with bovine serum albumin as a standard. Both assays provided similar concentrations.

Helicase Assays

The helicase activity of 4A was measured under two conditions, A and B, at either 4 or 18 °C. The reaction buffer consisted of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10% glycerol. All reactions were as described below, except where specified otherwise.

**Condition A—**4A protein (10 μM) was incubated for 1 min with 2 mM dTTP and 3 mM MgCl₂ in reaction buffer. The reactions were started by adding the DNA substrate mix, which consisted of 5 mM forked DNA, 10 mM dTTP, and 17 mM MgCl₂ in reaction buffer, to the preincubated protein.

**Condition B—**4A protein (8 μM) was incubated for 75 min at room temperature with 5 mM DNA substrate, 1.5 mM EDTA, and 4 mM dTTP in reaction buffer. The reactions were started by adding an equal volume of 11 mM MgCl₂ in reaction buffer.

Radiometric Helicase Assay

Reactions were carried out under conditions A or B in a 4 °C room and a FTC-100™ Programmable Thermal Controller (MJ Research, Inc.) to control the temperature of the reaction to ±0.1 °C. At various times after the start of the reaction, 5-μl aliquots were removed and quenched by addition of the quench buffer (100 mM EDTA, 3% SDS, 1% bromphenol blue, and 10% glycerol). The quenched samples (12 μl) were loaded immediately on native 10% polyacrylamide gels containing TBE (acrylamide/bisacrylamide = 19/1), and electrophoresis was started immediately after sample loading to prevent reannealing of DNA substrates. Electrophoresis conditions were 10 V/cm and 12 °C. A gel running temperature of 4 °C was chosen for forked DNA species to which short oligodeoxynucleotides were annealed to prevent DNA melting during electrophoresis. After electrophoresis, the gels were fixed in 10% (v/v) acetic acid, dried on Bio-Rad sequencing cell filter paper (2 h at 80 °C), exposed to phosphor storage screens (48 h), and quantitated using a PhosphorImager 445 SI (Molecular Dynamics, Inc.). Controls were run on each gel. The zero controls consisted of complete reaction mixtures minus dTTP. Controls to measure the extent of maximum unwinding were prepared by heat denaturing the DNA for 5 min at 95 °C and loading on the gel immediately.

RESULTS

Radiometric and Fluorimetric Assays for the Measurement of DNA Unwinding—The helicase activity of T7 DNA helicase 4A protein was quantitated by measuring the rate of ssDNA formation from dsDNA under single-turnover reaction conditions (excess protein over DNA). The DNA substrates consisted of a constant 33-bp duplex region and noncomplementary 5'- and 3'-tails of varying lengths (Fig. 1). Two assays were used to measure the single turnover kinetics of DNA unwinding, a discontinuous radiometric assay and a continuous fluorimetric assay.

In the radiometric assay, one of the strands of the forked DNA substrate was radiolabeled with 32P at the 5'-end for detection and quantitation. After stopping the reactions, the ssDNA products were resolved from the substrate forked DNA by native polyacrylamide gel electrophoresis, as shown in Fig. 2A, and quantitated using a PhosphorImager. In the continuous fluorimetric assay, DNA unwinding was measured in real time on a stopped-flow instrument by monitoring the change in

| DNA Concentrations were determined spectroscopically in TBE buffer (containing 8 mM urea) by taking absorbency measurements at λ = 260 nm in a Hewlett-Packard 8452A diode array spectrophotometer. The extinction coefficients for each DNA species were calculated from the DNA sequences using extinction coefficients of bases (liter · mol⁻¹ · cm⁻¹): ε₁ = 15200, ε₁₂ = 7050, ε₁₂₀ = 12010, ε₃ = 8400. |  |
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| Enzymes | Bacteriophage T7 gene 4A protein was expressed and purified as described previously (24). 4A is a M64L mutant of the 4A protein; the Met-64 is the initiation codon for the 4B protein. By mutating Met-64 to Leu, we were able to express 4A alone by preventing synthesis of 4B (25). The concentrations of purified proteins were determined both from absorbency measurements (ε₂₆₀ = 76,100 M⁻¹ · cm⁻¹) and by the Bradford method (26) with bovine serum albumin as a standard. Both assays provided similar concentrations. |
| Helicase Assays | The helicase activity of 4A was measured under two conditions, A and B, at either 4 or 18 °C. The reaction buffer consisted of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10% glycerol. All reactions were as described below, except where specified otherwise. |
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| **Condition B—** | 4A protein (8 μM) was incubated for 75 min at room temperature with 5 mM DNA substrate, 1.5 mM EDTA, and 4 mM dTTP in reaction buffer. The reactions were started by adding an equal volume of 11 mM MgCl₂ in reaction buffer. |
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Fig. 1. Synthetic forked-DNA substrates. The forked-DNA substrates, used to measure the helicase activity of 4A' protein, contained a 33-bp dsDNA region and two noncomplementary ssDNA tails. 5'- or 3'-tail, of lengths a and b, respectively. The fork junction is indicated by vertical bars in the sequences and in the cartoon. The sequence of the DNA strands in the largest substrate, the (65/27) forked DNA, is shown. The 5'-strand of the (65/27) substrate has a ssDNA tail of 65 bases, and the 3'-strand has a ssDNA tail of 27 bases. The bases comprising the 33-bp ds region are underlined in the sequence. Substrates containing gapped dsDNA in either the 5'- or the 3'-tail (a' or b', respectively) have a short oligodeoxynucleotide (gray bars) annealed to the end of the appropriate tail, leaving a ss region between the fork junction and the dsDNA end of the respective tail. The star in the cartoon indicates a possible position of the covalently attached fluorescein moiety or the 32P label.

![Image](68x584 to 288x729)

Fig. 2. Fluorimetric and radiometric helicase assays. A, shows a representative PhosphorImager scan of the native 12% polyacrylamide gel used to separate the radiolabeled forked-DNA substrate from the ssDNA product. DNA unwinding was assayed using the 27/27 forked DNA under condition A at 4 °C. Lane N represents unreacted dsDNA substrate, and lane H represents the heat-denatured substrate corresponding to ssDNA. B shows the kinetics of DNA unwinding measured using the fluorimetric stopped-flow assay. The filled diamonds represent the data for unwinding of the same substrate using the radiometric quenched-flow assay. Both methods provided comparable unwinding rates, 45 and 40 bp/s, respectively.

Fluorescein fluorescence. The fluorescein was linked covalently to either the 5'- or the 3'-strand of the forked DNA, such that the probe lay close to the duplex region (Fig. 1). Upon strand separation there was a 10% increase in the fluorescence of fluorescein, which provided a strong signal for continuous measurement of the helicase activity as shown in Fig. 2B. The fluorimetric stopped-flow assay devised here is simpler than similar continuous assays, based on the principle of fluorescence resonance energy transfer, reported in the literature (27–29). The assay reported here is more convenient since it requires only one strand of the DNA substrate to be modified. Although the stopped-flow assay for measuring DNA unwinding is more precise, it should be recognized that it is an indirect assay. Hence, it is important to check that both the radiometric and the fluorimetric assays provide comparable DNA unwinding kinetics. Such comparisons were conducted from time to time throughout the study, and both assays always provided the same kinetics of unwinding. One such comparison is shown in Fig. 2B, which shows that both the quenched flow radiometric assay and the stopped-flow assay provide comparable DNA unwinding rates, 45 and 40 bp/s, respectively.

T7 Helicase Interacts with both the 5'- and 3'-Strands during Initiation and Continued DNA Unwinding—It is known that T7 helicase requires both the 5' and 3' ssDNA tails for initiation of unwinding (10). However, it is not known whether the helicase interacts with one or both strands during DNA unwinding. It is possible that the helicase interacts with only one of the strands during DNA unwinding, and the interaction with the ssDNA tail in the other strand is required for initiation alone. An experiment was designed to test this idea. We prepared a (27/16) forked DNA substrate with a nick in the 3'-strand placed halfway in the duplex region by annealing three oligodeoxynucleotides (Fig. 3, substrate I). Unwinding beyond the nick would suggest that the 3'-tail is required for initiation alone. The results of the unwinding assay (Fig. 3) showed that 4A' cannot unwind beyond the nick. The same results were obtained when the nick was placed at an analogous position in the 5'-strand (data not shown). We conclude from those experiments that the helicase interacts with both the 3'- and 5'-strands during initiation and continued unwinding. Therefore, the mechanism of DNA unwinding by 4A' must involve interactions with both strands.

T7 DNA Helicase Requires Distinct Lengths of the 5'- and 3'-DNA Tails—To understand the interactions of the helicase with the ssDNA tails during initiation of unwinding, a detailed...
Interactions of the T7 DNA Helicase with the Forked DNA

**Fig. 4. The dependence of the helicase activity of 4A’ on the 5’- and 3’-tail lengths measured under condition A (illustrated in the schematic above the graphs).** A shows the dependence of the helicase activity on the 5’-tail length (filled circles, length $a$ of the 5’-tail is varied, and the 3’-tail is 27 bases long), and the 3’-tail length (filled triangles, length $b$ of the 3’-tail is varied, and the 5’-tail is 27 bases long). The helicase activity (bp/s) was measured at 18 °C under condition A using the fluorimetric assay on a stopped-flow instrument. The solid line through the filled circles represents the fit of the 5’-tail length dependence data to a hyperbola. B shows the dependence of the helicase activity on the 5’- and 3’-tail lengths, measured at 4 °C under condition A, using the radiometric assay. The solid line through the filled circles represents the fit of the 5’-tail length dependence data to a hyperbola, and the inset shows an extrapolation of the hyperbola to longer 5’-tail lengths.

The 3’-tail length dependence was investigated using forked DNA substrates with a fixed 27-nucleotide 3’-tail and increasing lengths of the 5’-tail. The results showed that (42/00), (42/05), and the (42/07) DNA substrates with 0, 5, or 7-nucleotide 3’-tails were poor substrates of the helicase (Fig. 4A). When the 3’-tail length was systematically increased from 0, 5, 7, 10, 13, 15, to 27 bases, the unwinding rate increased sharply and reached a maximum at around 13 bases of 3’-tail. The maximum rate of unwinding under condition A at 18 °C with the (42/13) fork was 11 bp/s.

The 5’-tail length dependence was investigated under the same condition as above using forked DNAs with a fixed 27-nucleotide 3’-tail and increasing lengths (10–65 bases) of the 5’-tail. As shown in Fig. 4A, the (10/27) substrate, with a 10-nucleotide 5’-tail, was a poor substrate of the helicase. As the 5’-tail length was increased from 10 to 65 nucleotides, the rate of unwinding increased, but the increase was more gradual as compared with the 3’-tail dependence. The unwinding rates kept increasing even when the 5’-tail was 65 nt long. The 5’-tail dependence data were fit to a hyperbola to obtain an estimate of the half-maximal 5’-tail length, which was determined as 65 nucleotides with a maximum unwinding rate of 12 bp/s.

It seemed unusual to us that optimum unwinding required a >65-nucleotide 5’-tail because it has been shown previously that each 4A’ hexamer binds and protects 25–30 bases of ssDNA (8, 9, 22). To ensure that the above dependence was not due to the indirect nature of the fluorimetric assay used to measure DNA unwinding, we repeated both the 5’- and 3’-tail dependences using the radiometric assay. Since the unwinding rates at 18 °C were too fast to measure manually, the radiometric assays were carried out at 4 °C (Fig. 4B). The 5’- and 3’-tail length dependences under these conditions were the same as shown in Fig. 4A. The optimum length of the 3’-tail was 15 nt, and the unwinding rate increased gradually as the 5’-tail was increased from 10 to 65 bases, and the rates did not saturate at 65 bases. When the 5’-tail dependence data were fit to a hyperbola, a half-maximal 5’-tail length of 114 bases and a maximum rate of unwinding at 4 °C equal to 0.5 bp/s were determined.

To investigate if the unusual 5’-tail dependence, observed in the experiments described above, was due to the initiation conditions used to measure the helicase activity, we explored alternative conditions to initiate DNA unwinding. Particularly, we wished to establish conditions in which we could preassemble the 4A’ hexamer on the DNA prior to measuring DNA unwinding. We have shown previously that 4A’ forms a stable complex with ssDNA in the presence of dTMP-PCP (8), the nonhydrolyzable analog of dTTP, which does not support DNA unwinding. We could therefore assemble the protein-DNA complex in the presence of Mg(dTMP)-PCP and initiate the reactions by adding excess Mg(dTTP). When 4A’ was assembled on the DNA in the presence of dTMP-PCP, the rate of DNA unwinding was greatly inhibited (data not shown). In the process of exploring alternative conditions, we found that 4A’ assembled into stable hexamers and also bound the forked DNA tightly in the presence of dTTP without Mg(II) (data not shown). Since no DNA unwinding was observed in the absence of Mg(II), we could now preassemble the 4A’-DNA complex in the presence of dTTP and add Mg(II) to initiate the reaction. dTTP hydrolysis in the absence of Mg(II) was found to be very slow. We refer to these assay conditions as condition B.

The 5’- and 3’-tail length dependence experiments were repeated under condition B at 18 °C using the continuous fluorimetric stopped-flow assay. 4A’ protein was preassembled on the forked DNA in the presence of dTTP and in buffer containing EDTA to chelate any contaminating metal ions, and the reaction was initiated by adding Mg(II). The dependence of the DNA unwinding rate with increasing 3’- and 5’-tail lengths is shown in Fig. 5. The 3’-tail length dependence, under condition B, remained unchanged from that observed under condition A (Fig. 4A). The same sharp increase in the unwinding rate with increasing 3’-tail length was observed, providing an optimal 3’-tail length of 15 nucleotides. Interestingly, the 5’-tail dependence under condition B was different from that observed under condition A (Fig. 4A). In contrast to the gradual increase in...
unwinding rate with increasing 5'-tail length under condition A, a sharp increase in DNA unwinding rate was observed under condition B. The rate increased sharply between 5'-tails lengths of 25–35 nt, remained constant up to a tail length of 42 nt, and actually decreased beyond a 40-nt long 5'-tail. The maximum rate of DNA unwinding under condition B was 66 bp/s, a close to 6-fold increase from the rates observed under condition A.

Protein Concentration Dependence of the Helicase Activity—DNA unwinding rates were measured as a function of increasing 4A' protein concentration, both under conditions A and B, to understand the reason for the differences in the unwinding rates and the 5'-tail length dependencies under the two conditions. Experiments were carried out at 18 °C with the (42/27) forked DNA, and unwinding was measured using the fluorimetric stopped-flow assay. The protein concentration dependence of the unwinding rates under conditions A and B are shown in Fig. 6. Under condition A, where the helicase was not preincubated with the forked DNA, the unwinding rates increased almost linearly with [4A'] and showed no trend toward saturation up to 12 μM 4A'. Under condition B, however, optimal DNA unwinding was achieved at about 2 μM 4A'. Since all the 5' and 3'-tail dependences (shown in Figs. 4 and 5) were measured using 4 μM 4A', the unwinding rates under condition A were limited by protein concentration, but the unwinding rates under condition B were in fact independent of protein concentration.

T7 DNA Helicase Requires ssDNA Regions in Both the 5'- and 3'-Tails for Initiation of Unwinding—The above results show that T7 helicase has distinct length requirements for the 5'- versus the 3'-tail. To investigate if 4A' requires specifically ssDNA tails for the helicase function, we have modified the structure of the 5'- and the 3'-tails. The tails were converted into partially dsDNA structures by annealing complementary DNAs of different lengths to the 3'- or the 5'-tail. The complementary DNAs were designed in such a way that the resulting forked DNAs had increasing lengths of ssDNA gaps near the fork junction (see Fig. 1). The ssDNA gaps in the 3'-tail ranged in length from 0, 5, 10, 13, 15, to 18 bases. Fig. 7 shows the unwinding rates measured under condition A as a function of the single-stranded gap length on the 3'-tail. DNA unwinding as well as the native gels were run at 4 °C to minimize thermal fraying of short dsDNA regions. As with the 3'-tail dependence, which is shown on the same figure for comparison, the unwinding rates increased as the ssDNA gap region increased in length from 0 to 15 nt, suggesting that the 3'-tail cannot be replaced by a ds-DNA. Replacement of the 3'-tail by three groups of hexaethylene glycol, equivalent in length to 15 bases of ssDNA, also resulted in undetectable helicase activity (data not shown). We conclude from these results that 4A' has a specific requirement for a ssDNA region in the 3'-tail. The same results were obtained when the 5'-tail was converted into partially dsDNA. Forked DNAs containing a gap of 20 bases ssDNA in the 5'-tail were not unwound, but DNAs with a gap of 40 bases ssDNA were unwound at maximal rates at 18 °C under condition B (data not shown).

FIG. 5. The dependence of the helicase activity on the 5'- and 3'-tail lengths under condition B. The helicase activity was measured under condition B (illustrated in the cartoon above the graph) at 18 °C using the fluorimetric assay. The unwinding rates (bp/s) are plotted against the lengths of the 5'-tail (filled circles, 27 bases 3'-tail) and the 3'-tail (filled triangles, 42 bases 5'-tail).

FIG. 6. 4A' protein concentration dependence of the helicase activity under conditions A and B. The rate of unwinding forked DNA substrates containing increasing lengths of ssDNA gap in the 3'-tail (27/27ssb') was measured at 4 °C using the radiometric assay under condition A (filled circles). The 3'-tail length dependence data (27b) under the same reaction conditions is shown for comparison (open triangles).

FIG. 7. Dependence of the helicase activity on the ssDNA gap length in the double-stranded 3'-tail. The rate of unwinding forked DNA substrates containing increasing lengths of ssDNA gap in the 3'-tail (27/27ssb') was measured at 4 °C using the fluorimetric assay under condition A (filled circles). The 3'-tail length dependence data (27b) under the same reaction conditions is shown for comparison (open triangles).

DISCUSSION

We have studied here the interactions of T7 DNA helicase with the forked DNA substrate with the goal of understanding the substrate requirement for initiation of DNA unwinding. It was known that T7 helicase required two noncomplementary ssDNA tails to initiate DNA unwinding (10). However, the nature of the interaction of the helicase with the forked DNA was not understood. The helicase could interact with either one or both ssDNA tails of the forked DNA, with the dsDNA alone, or with both ds- and ssDNA. It is known that T7 helicase binds preferentially to ssDNA, and its interaction with the dsDNA is very weak. In addition, at least one of the DNA-binding sites is within the central hole of the hexameric ring (9, 22, 30). Since the diameter of the central hole is 20–25 Å, it could accommodate a dsDNA or a ssDNA. However, binding of the dsDNA within the central hole was not observed in the electron microscopy studies (9), suggesting that one of the two noncomplementary ssDNA tails of the forked DNA is bound within the hole. If T7 helicase translocates on ssDNA in the 5' → 3' direction as studies in the literature suggest (31–33), and if the
helicase maintains the same directionality of translocation during DNA unwinding, then the helicase must bind ssDNA in the 5'-strand through the hole. This idea is consistent with recent studies (34), which showed that a covalent benzo[a]pyrene-DNA adduct in the 5'-strand inhibited unwinding by T7 helicase, but benzo[a]pyrene in the 3'-strand had no effect on the unwinding activity.

We have shown that T7 DNA helicase requires both the 5'- and the 3'-noncomplementary DNA tails for initiation of DNA unwinding, and unwinding was greatly inhibited when one of the tails was removed. Note that in vivo the ssDNA regions required for initiation of DNA unwinding are likely provided by the priming proteins involved in DNA replication (35). Whereas a number of studies suggest that the helicase may bind and move on the 5'-tail, the role of the 3'-tail or the nature of its interaction with the helicase is not clear. It is possible that a noncomplementary 3'-tail is required simply because the 3'-strand needs to be excluded from the central hole during DNA unwinding, i.e. the helicase is acting as a wedge between the two DNA strands. If a noncomplementary 3'-tail is not present then the dsDNA or the ss/dsDNA junction may bind in a non-productive manner in the hole and inhibit DNA unwinding. To test this hypothesis, we converted the 3' ssDNA tail into a dsDNA tail. If the helicase does not interact specifically with the ssDNA region in the 3'-tail, then the forked DNA containing the ssDNA tail should be unwound by the helicase because the dsDNA tail would be excluded from the hole. Our results showed that conversion of the 3' ssDNA tail into a dsDNA tail greatly inhibited the unwinding activity. The same result was seen when the 5' ssDNA tail was converted into a dsDNA tail. We conclude that T7 helicase must interact with both the ssDNA tails during initiation of DNA unwinding. Experiments with nicked DNA substrate showed that the helicase maintains this interaction with the two ssDNA strands also during DNA unwinding.

Systematic investigation of the unwinding activity as a function of 5'- and 3'-tail lengths showed that T7 helicase requires different lengths of 5'- and 3'-tails for optimal DNA unwinding. The optimum length of the 3'-tail under all conditions was found to be 15 bases, whereas the optimum length of the 5'-tail ranged from 35 bases to >65 bases. These results lead us to believe that the helicase interacts asymmetrically with the two tails of the forked DNA during DNA unwinding. Under condition A, where DNA unwinding was started by mixing the helicase hexamer with the forked DNA, a >65-nt long 5'-tail was required to achieve optimal unwinding rates. This result was puzzling, since previous studies have shown that each helicase hexamer binds and protects 25–30 bases of ssDNA inside the central hole (9, 22). The requirement for a longer 5'-tail suggests that multiple hexamers might be required for optimal DNA unwinding, since two hexamers bind to a ssDNA 65 bases long (9, 22). Additional DNA unwinding experiments under condition B, however, proved this not to be true. In fact, under condition B, where the helicase was preassembled on the DNA, unwinding rates decreased beyond 40 bases of 5'-tail, suggesting that multiple hexamers, when bound to the 5'-tail, are inhibitory.

Under condition B, optimal unwinding rates were reached with a 5'-tail length of 35 bases, which is consistent with one hexamer DNA-binding site of 25–30 bases. The reason for an apparent requirement of a long 5'-tail under condition B is not entirely clear. The unwinding rates under condition B increased with increasing protein concentration and with increasing 5'-tail length above 35 bases. Thus, it is possible that the rate-limiting association of the helicase hexamer with the DNA is responsible for the apparent requirement of a long 5'-tail. The protein dependence study suggested that 25–30 μM 4A'-protein would be required to achieve the 60 bp/s unwinding rates observed under condition B. Perhaps the optimal 5'-tail length would be closer to 35 bases, if the experiments under condition A were carried out at a saturating 4A' concentration. However, due to the high protein concentrations required to conduct such an experiment, we have not tested this idea. The present studies have established the optimal forked DNA substrate and the reaction conditions necessary to measure DNA unwinding. The average unwinding rates under optimal conditions were measured to be close to 60 bp/s at 18 °C, which approached 260 bp/s at 30 °C (data not shown). This rate is very close to the measured speed of 300 s⁻¹ for replication fork movement at 30 °C (36) and suggests that the helicase is highly efficient by itself in unwinding duplex DNA.

Our model for how T7 DNA helicase interacts with the forked DNA is shown in Fig. 8. The hexamer binds to the 5'-tail through the central hole, whereas the 3'-tail is excluded from the central hole and interacts at a separate site on the helicase, perhaps in the front or on the outside of the hexamer ring as shown in Fig. 8 (arrow). We propose that T7 helicase uses an exclusion mechanism for DNA unwinding, where the NTPase activity fuels the unidirectional movement of the helicase on the 5'-strand, bound in the hole of the hexamer, and the 3'-strand is excluded from the central hole, and interactions with both strands lead to dsDNA separation.

Several models of helicase-catalyzed DNA unwinding have been proposed in the literature (4, 5, 37). In general these models have been divided into passive or active mechanisms. In the passive mechanism, the helicase is thought to unwind DNA by occupying the ssDNA regions generated by thermal melting of duplex DNA. In one model of a passive mechanism, the helicase does not move unidirectionally on the DNA; hence, to melt long stretches of DNA a number of helicase molecules would have to bind to the ssDNA generated by thermal fraying. In another model, the helicase does move unidirectionally, but upon interaction with the dsDNA it waits for thermal fraying for strand separation. Thermal fraying itself appears to be a fast process (38). But it is not clear if thermal fraying can
Interactions of the T7 DNA Helicase with the Forked DNA

separate the dsDNA bases far enough for the helicase to bind and unwind DNA at speeds required for DNA replication, repair, and recombination. Several active mechanisms, where the helicase moves unidirectionally on the DNA, have been proposed. Some of these have been classified into inchworm, rolling, or torsional models by Lohman (5). In the rolling model of unwinding, proposed for rep helicase, the helicase interacts with both the ss- and dsDNA, and DNA strands are separated upon binding to the dsDNA (39). Since T7 DNA helicase prefers to interact with ssDNA, we believe that it does not use a rolling-type mechanism, but DNA unwinding by the exclusion model proposed above could occur by an inchworm or the torsional mechanism. In the inchworm mechanism, the helicase would translocate unidirectionally on the 5′-tail and cause DNA unwinding by simply excluding the 3′-tail from the central hole. Thus, DNA unwinding can occur without direct interactions of the helicase with the duplex DNA. The exclusion model proposed here is not limited to hexameric helicases. All that is required is that the two ssDNA strands are bound at separate DNA sites on the helicase so that the helicase can act as a wedge. In the torsional mechanism, the helicase interacts with the ssDNA in both strands. The translocation and rotation of the helicase on one strand would result in rotation of the second strand around the first, leading to unwinding of the dsDNA by the torque on the two strands. Whereas the exact mechanism of DNA unwinding by T7 helicase or other helicases is not known, based on available data we propose that T7 helicase may use a combination of the inchworm and torsional mechanisms to unwind DNA.

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