

LETTERS

DNA synthesis provides the driving force to accelerate DNA unwinding by a helicase

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Helicases are molecular motors that use the energy of nucleoside 5'-triphosphate (NTP) hydrolysis to translocate along a nucleic acid strand and catalyse reactions such as DNA unwinding. The ring-shaped helicase¹ of bacteriophage T7 translocates along single-stranded (ss)DNA at a speed of 130 bases per second²; however, T7 helicase slows down nearly tenfold when unwinding the strands of duplex DNA³. Here, we report that T7 DNA polymerase, which is unable to catalyse strand displacement DNA synthesis by itself, can increase the unwinding rate to 114 base pairs per second, bringing the helicase up to similar speeds compared to its translocation along ssDNA. The helicase rate of stimulation depends upon the DNA synthesis rate and does not rely on specific interactions between T7 DNA polymerase and the carboxy-terminal residues of T7 helicase. Efficient duplex DNA synthesis is achieved only by the combined action of the helicase and polymerase. The strand displacement DNA synthesis by the DNA polymerase depends on the unwinding activity of the helicase, which provides ssDNA template. The rapid trapping of the ssDNA bases by the DNA synthesis activity of the polymerase in turn drives the helicase to move forward through duplex DNA at speeds similar to those observed along ssDNA.

The DNA factory of bacteriophage T7 is one of the simplest and most widely used model systems for studying replication mechanisms⁴. The T7 replication complex, containing a helicase (T7 gp4), a DNA polymerase (T7 gp5 in complex with *Escherichia coli* thioredoxin) and a ssDNA-binding protein (T7 gp2.5), efficiently catalyses leading and lagging strand DNA synthesis⁵. T7 DNA

polymerase (polymerase-thioredoxin) alone can elongate a DNA primer when the downstream DNA template is single stranded (Fig. 1a). The average rate of DNA synthesis by T7 DNA polymerase increases in a hyperbolic manner with dNTP concentration, with a $K_{1/2}$ of 11 μM and V_{max} of 230 nucleotides per second at 18 °C (Fig. 1b), which is consistent with previous pre-steady-state kinetic measurements⁶. DNA synthesis is blocked when the downstream template DNA is duplex (Fig. 1c). T7 DNA polymerase incorporates only 4–5 nucleotides on the duplex template before DNA synthesis stalls. These results indicate that T7 DNA polymerase cannot unwind the duplex DNA beyond 4–5 base pairs (bp) and hence cannot catalyse strand displacement DNA synthesis.

T7 helicase uses the energy of dTTP hydrolysis for translocation and unwinding of duplex DNA^{3,7–9}. Using an all-or-none radiometric assay carried out under single-turnover conditions¹⁰, we measured the unwinding activity of T7 helicase on a 30-bp replication substrate (Fig. 2a, b). T7 helicase was pre-incubated with replication substrate (Fig. 2a) in the presence of dTTP without Mg²⁺ (conditions that allow assembly of the protein on the DNA, but no unwinding), and the reaction was started by rapid addition of Mg²⁺. T7 helicase unwinds the replication substrate at an average rate of 9 bp s⁻¹ in the absence of T7 DNA polymerase (Fig. 2b). In the presence of T7 DNA polymerase, saturating dTTP and with the other three dNTPs at 100 μM—conditions under which T7 DNA polymerase is capable of incorporating nucleotides at its maximal rate—the unwinding rate of T7 helicase was 114 bp s⁻¹ (Fig. 2b, c). This is about a 13-fold enhancement in the unwinding rate of T7 helicase.

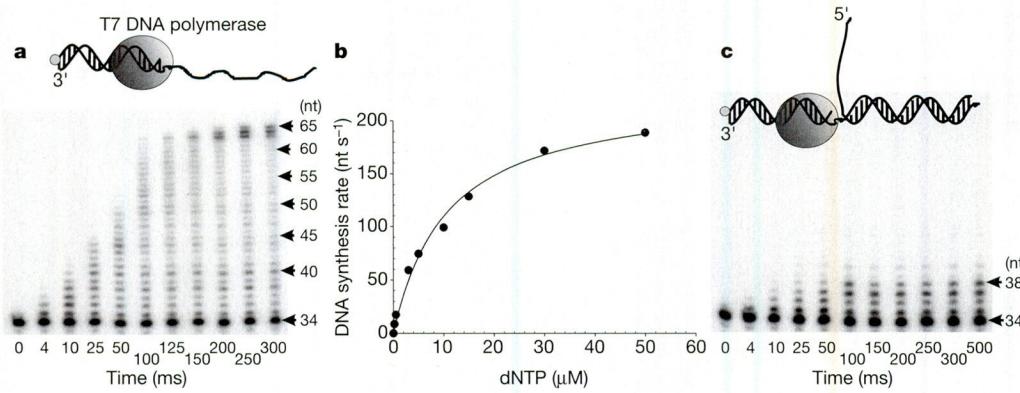


Figure 1 | DNA synthesis by T7 DNA polymerase. **a**, Progressive elongation of the radiolabelled 34-nucleotide primer annealed to the 65-nucleotide template at a dNTP concentration of 100 μM. **b**, The average DNA synthesis rate increases hyperbolically with dNTP concentration (observed

rate = $V_{max}[\text{dNTP}] / (K_{1/2} + [\text{dNTP}])$ with $K_{1/2} = 11 \pm 2 \mu\text{M}$ and $V_{max} = 228 \pm 11$ nucleotides per second. **c**, Elongation of the 34-nucleotide primer on the 30-bp replication substrate. nt, nucleotides.

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We find that the helicase rate of stimulation depends on the rate of DNA synthesis, which in turn depends on the concentration of dNTP (Fig. 1b). T7 helicase uses only dTTP as an energy source for unwinding (dissociation constant (K_d) of approximately 80 μM)³, and other dNTPs do not support DNA unwinding⁸. To investigate the effect of DNA synthesis rate on helicase rate of stimulation, we varied the concentration of three dNTPs while keeping dTTP at 1 mM. The observed unwinding rate increases with increasing dNTP concentration in a hyperbolic fashion, with a $K_{1/2}$ identical to that for DNA synthesis (Fig. 1b) and V_{\max} of 130 bp s⁻¹ (Fig. 2c). These results show that the unwinding rate of T7 helicase depends on the speed of T7 DNA polymerase, and when the DNA polymerase can incorporate nucleotides at a rate much faster than the translocation rate of the helicase, it stimulates the helicase to unwind DNA at the same rate as its translocation along ssDNA (130 nt s⁻¹)².

The coupled action of the helicase and polymerase can be monitored by the primer elongation assay. We show that the 30-bp DNA substrate is not replicated efficiently by T7 DNA polymerase alone (Fig. 1c); however, T7 DNA polymerase replicates this substrate efficiently in the presence of T7 helicase (Fig. 3a). The time course of base additions shows that about 18 nucleotides are added in 150 ms. This corresponds to a maximum rate of DNA synthesis of about

120 nucleotides per second by the helicase–polymerase complex. This rate is comparable to the one measured by the all-or-none unwinding assay (Fig. 2b, c). Thus, both the DNA unwinding assay and the DNA synthesis assay show that T7 DNA polymerase activates T7 helicase.

The activities of T7 helicase and T7 DNA polymerase are interdependent. The helicase provides the DNA polymerase with the ssDNA template for DNA synthesis. Similarly, the DNA polymerase serves to increase the unwinding rate of the helicase. In addition, the experiments show that the rate of DNA synthesis dictates the stimulated unwinding rate. What is the mechanism of this stimulation? Because T7 helicase and T7 DNA polymerase specifically interact to form a complex, one possibility is that the rate increase occurs through an allosteric change in the helicase producing a better unwinding motor.

T7 helicase forms a specific complex with T7 DNA polymerase through 17 carboxy-terminal amino acid residues¹¹. To test the function of the specific complex formation, we prepared a mutant T7 helicase that lacked these 17 C-terminal residues (ΔCt mutant). The ΔCt mutant of T7 helicase has both wild-type ssDNA-stimulated dTTPase and helicase activities¹¹, translocates along ssDNA with a rate similar to wild-type helicase (data not shown), but does not form a stable complex with the polymerase¹¹. Rapid DNA synthesis was observed in the presence of the ΔCt helicase mutant (Fig. 3b). On the basis of the addition of 16 nucleotides in 100 ms, we estimate that the 30-bp duplex is replicated with a rate of about 160 nucleotides per

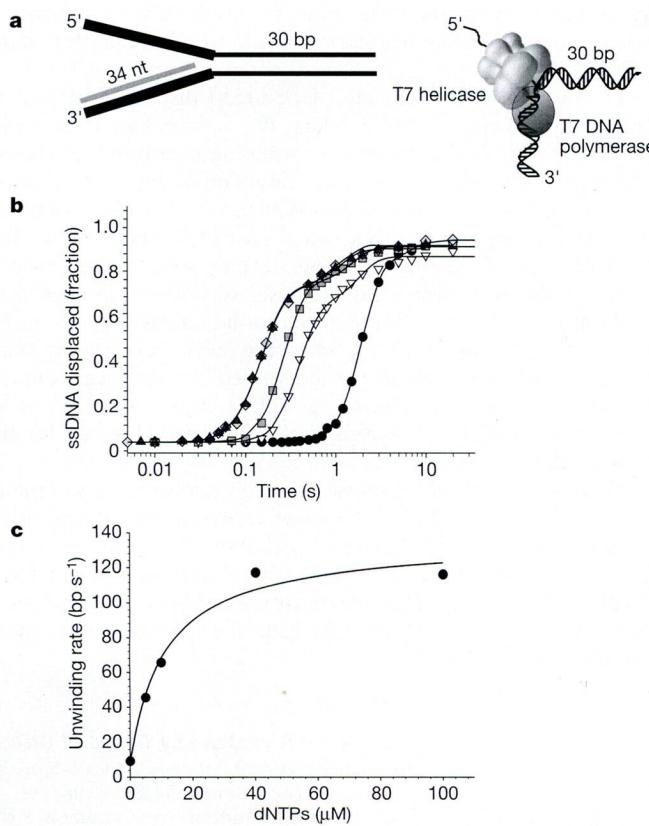


Figure 2 | The unwinding rate of T7 helicase depends on the speed of DNA synthesis. **a**, 30-bp replication substrate. **b**, Unwinding kinetics of the 30-bp region of the replication substrate in the absence (circles) or presence of T7 DNA polymerase at 1 mM dTTP and at 5 μM (inverted white triangles), 10 μM (squares), 40 μM (filled triangles) and 100 μM (diamonds) for each of the other three dNTPs. An unwinding rate (sk) of 9.3 bp s⁻¹ (80% amplitude) and 2.6 bp s⁻¹ (20%) was obtained in the absence of T7 DNA polymerase by fitting the kinetics to equation (1) ($s = 3.6 \pm 0.15 \text{ bp step}^{-1}$; $k_1 = 2.6 \pm 0.1 \text{ steps s}^{-1}$; $k_2 = 0.7 \pm 0.1 \text{ steps s}^{-1}$). In the presence of T7 DNA polymerase and a dNTP concentration of 100 μM , the unwinding rate is 114 bp s⁻¹ (90%) and 15 bp s⁻¹ (10%) ($s = 5.3 \pm 0.4 \text{ bp step}^{-1}$; $k_1 = 22 \pm 1.6 \text{ steps s}^{-1}$; $k_2 = 3 \pm 0.4 \text{ steps s}^{-1}$). **c**, The unwinding rate increases hyperbolically with dNTP concentration, with $K_{1/2} = 11 \pm 4 \mu\text{M}$, and provides a maximum unwinding rate of $127 \pm 12 \text{ bp s}^{-1}$.

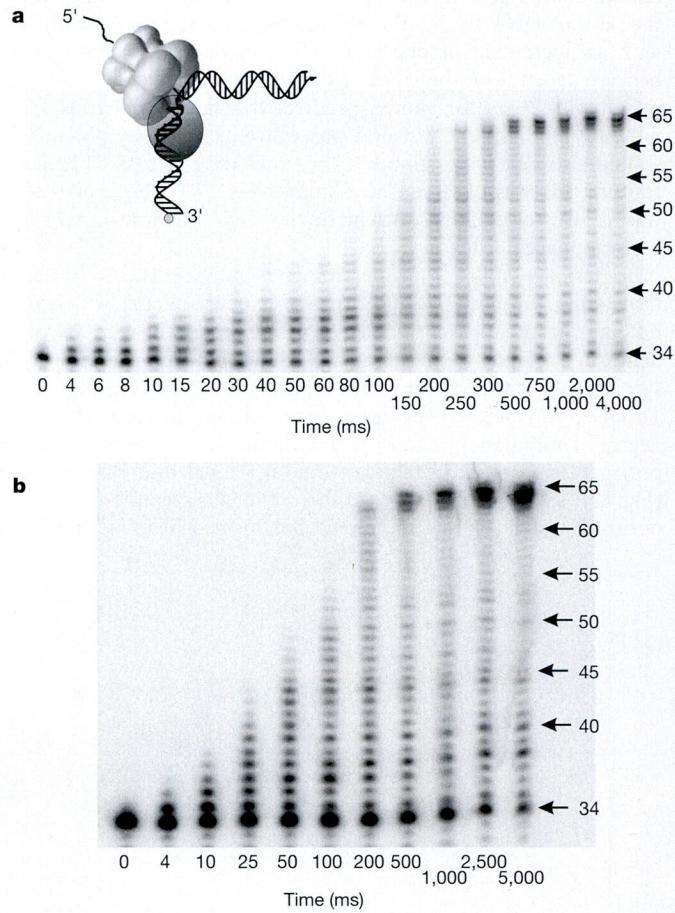


Figure 3 | DNA synthesis by T7 DNA polymerase and T7 helicase. **a**, Progressive elongation of the 34-nucleotide primer by T7 DNA polymerase and T7 helicase on the 30-bp replication substrate. **b**, Kinetics of DNA synthesis by T7 DNA polymerase and the ΔCt T7 helicase mutant on the 30-bp replication substrate.

second. Therefore, the interaction between T7 DNA polymerase and the C-terminal residues of T7 helicase is not necessary for stimulation of the helicase reaction. Of note, the interaction between T7 helicase and T7 DNA polymerase serves to increase the processivity of the complex, which becomes critical for copying long DNA templates¹¹. T7 helicase and T7 DNA polymerase possess a sufficient degree of intrinsic processivity to unwind and replicate the short DNA duplexes used here, in the absence of specific interactions involving the C-terminal residues of the helicase. A smaller fraction of DNA primer was elongated initially with the ΔC_T T7 helicase, indicating that specific interactions also have a function in the initial complex assembly.

Any protein that binds tightly to ssDNA can shift the equilibrium of the DNA unwinding reaction ($\text{dsDNA} \rightleftharpoons \text{two ssDNAs}$) towards ssDNA¹². Therefore, the DNA polymerase might facilitate the helicase unwinding activity by trapping the complementary strand that is displaced by the helicase. Consequently, we investigated whether ssDNA-binding proteins would have the same effect. We used a fork DNA as an unwinding substrate that contained a T₁₅ 3' ssDNA tail, as well as T7 gp2.5 (ref. 13) and *E. coli* SSB¹⁴ proteins that bind ssDNA with high affinity. The results (Supplementary Fig. 1) show that the ssDNA-binding proteins have little effect on the unwinding rate. A modest increase in the initial unwinding amplitude was observed, which suggests that the processivity of unwinding is higher in the presence of the ssDNA-binding proteins.

The experiments with ssDNA-binding proteins indicate that helicase stimulation by T7 DNA polymerase must involve more than simply binding of the protein to the displaced complementary strand. Although both DNA polymerase and SSB proteins capture the nascent DNA strand, the polymerase does so more rapidly and with an increment of one base. The fact that stimulation of T7 helicase depends on the speed of the DNA polymerase substantiates this hypothesis. Our model predicts that a heterologous DNA polymerase that is as fast and processive as T7 DNA polymerase should also be able to stimulate the unwinding rate of T7 helicase. Indeed, this was observed in a study where T7 DNA polymerase was shown to support efficient duplex DNA replication with the bacteriophage T4 helicase¹⁵.

To test the ability of a heterologous polymerase to stimulate unwinding, we used T4 gp43 core DNA polymerase, which does not have strand displacement activity (Fig. 4a). In the presence of T7 helicase, T4 DNA polymerase catalyses strand displacement DNA synthesis at a rate of about 30 nucleotides per second (Fig. 4b). Thus, T4 DNA polymerase provides a threefold rate increase. The lower degree of stimulation is consistent with the slower speed of T4 DNA polymerase on a ssDNA template (about 50 nucleotides per second) (Fig. 4c). To compensate for the different intrinsic synthesis rates, the optimal stimulated rate of the T4 polymerase–helicase should be

compared with the rate of T7 polymerase–helicase measured at 1.8 μM (approximately 50–60 nucleotides per second, compare Fig. 4e and c), the conditions providing the same intrinsic synthesis rate. Under these conditions, T7 DNA polymerase stimulates T7 helicase to the same extent as T4 DNA polymerase (compare Fig. 4d and b). The fraction of DNA primer elongated to full-length product is less with the heterologous T4 DNA polymerase, consistent with the idea that specific interactions are important for initial complex assembly. These results indicate that a heterologous DNA polymerase and helicase can show cooperativity in DNA synthesis and unwinding, and that the extent of the helicase activity stimulation depends upon the intrinsic rate of DNA synthesis.

The reduced translocation rate of the helicase during unwinding and its stimulation by polymerases can be explained in terms of a brownian motor mechanism^{16–18}. The brownian motor (helicase) translocates along the DNA strand through a succession of power strokes and diffusion states. While in the diffusion state the helicase can move in either direction along the DNA; the power strokes make the net movement unidirectional. In the presence of the complementary strand, DNA base pairs fluctuating between closed and open states produce a net force directed against the helicase forward motion. The power strokes of the helicase can overcome the force; however, during the diffusion phase, backward motion occurs frequently. Therefore, the observed DNA unwinding rate is slower than the helicase's intrinsic translocation rate along ssDNA³.

T7 and T4 DNA polymerases lack strand displacement activity (unwinding) beyond 4–5 bp. Thus, the activities of these DNA polymerases are dependent on the unwinding activity of the helicase, which provides a ssDNA template. The action of the DNA polymerase (that is, the formation of new duplex DNA) in turn results in efficient trapping of the DNA strand created by the helicase. The forward steps of the DNA polymerase may serve to increase the helicase's net forward movement (push) or decrease the helicase's backward slips (brake). The observed unwinding rate depends on the speed of DNA synthesis. Only when the polymerase rapidly traps the ssDNA bases as soon as they are formed by the helicase can the helicase obtain its maximal rate, and the complex is able to move through duplex DNA at speeds similar to those observed for the helicase along ssDNA.

The synergy between helicase and polymerase is important in DNA replication, and is also evident from studies of replication complexes of *E. coli*^{19,20}, human mitochondria²¹ and bacteriophage T4^{15,22,23}. Studies of the simpler T7 system continue to provide a deeper understanding of the enzymatic mechanisms of DNA replication, many of which are likely to be generally applicable to the more complex replication systems.

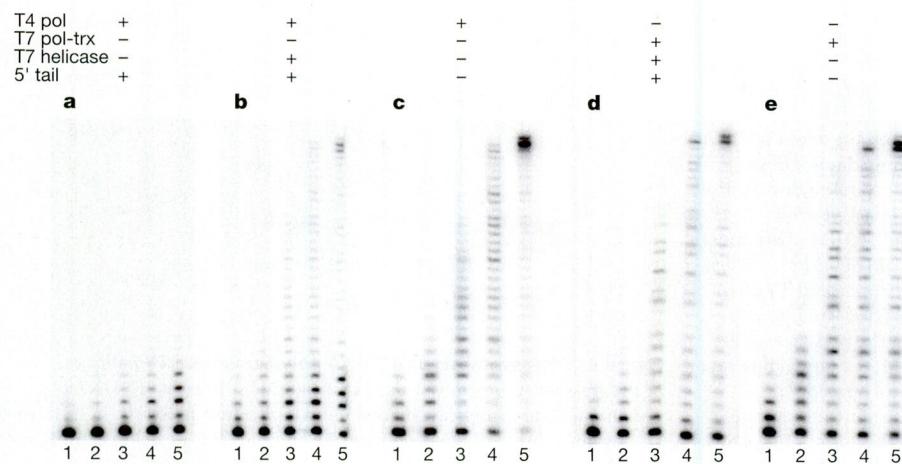


Figure 4 | DNA synthesis by T4 and T7 DNA polymerases and T7 helicase. Lanes 1–5 in all experiments correspond to reaction times of 0.025, 0.1, 0.5, 1.0 and 3.0 s, respectively. **a**, DNA synthesis by T4 DNA polymerase on the 30-bp replication substrate at 100 μM for each dNTP. **b**, DNA synthesis by T4 DNA polymerase and T7 helicase on the replication substrate, at 1 mM dTTP and 100 μM for each of the other three dNTPs. **c**, DNA synthesis by T4 DNA polymerase on the substrate with a ssDNA template at 100 μM for all dNTPs. **d**, DNA synthesis by T7 DNA polymerase (polymerase-thioredoxin or pol-trx) and T7 helicase on the replication substrate at 1 mM dTTP and 1.8 μM for each of the three other dNTPs. **e**, DNA synthesis by T7 DNA polymerase on the substrate with a ssDNA template at 1.8 μM for each dNTP.

METHODS

DNA. Oligodeoxynucleotides (Integrated DNA Technologies) were purified by polyacrylamide gel electrophoresis (PAGE), and DNA concentrations were determined in 8 M urea (260 nm absorbance and calculated extinction coefficients). DNA was radiolabelled at the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase. The replication substrate was made by annealing the 5' strand (5'-T₃₅-GAGCGGATTACTATACATTAGAATTCA), 34-nucleotide primer (5'-CTAGTTACAGAGTTATGGTGACGATAACAACTAT) and the 3' strand (3'-GATCAATGTCTCAATACCACTGCTATGTTGATATCTGCC TAATGATATGATGTAATCTTAAGT). The fork DNA was made by annealing the 5' strand with the T₁₅ 3' strand (3'-T₁₅-CTCGCCTAATGATATGATGT AATCTTAAGT).

Proteins. T7 helicase gp4A', a M64L mutant of T7 helicase-prime protein, was purified as described previously⁹. T7 gp5 (D5A, E7A, exo⁻ gp5) was purified as described⁶ and *E. coli* thioredoxin was purchased from Sigma Chemicals. The Δ Ct T7 helicase was prepared as described¹¹. T7 gp2.5 was purified as described¹³, *E. coli* SSB was a gift from M. O'Donnell, and T4 DNA polymerase exo⁻ mutant (D219A)²⁴ was a gift from A. Berdis. Protein concentrations were determined by absorbance measurements at 280 nm in 8 M urea using their calculated extinction coefficients.

DNA unwinding kinetics. Unwinding kinetics were measured in a RQF3 rapid quench-flow instrument (KinTek) at 18°C (ref. 3). T7 helicase, DNA substrate (with radiolabelled 5'-strand), 2 mM dTTP and 3 mM EDTA in buffer T (50 mM Tris-Cl, pH 7.5, 40 mM NaCl, 10% glycerol) was loaded in one syringe of the quench-flow apparatus. MgCl₂ (final free concentration of 4 mM), dNTPs (various concentrations) and trap (3 μ M of unlabelled 5' strand) was added from the second syringe to initiate the reaction. The reactions were quenched after pre-determined times, resolved by native PAGE, and products analysed as described previously³. The unwinding reactions contained a final 200 nM T7 helicase hexamer and 2.5 nM of 30-bp replication substrate. The unwinding reactions with 200 nM T7 DNA polymerase contained 2 μ M *E. coli* thioredoxin, 200 nM T7 helicase and 100 nM replication substrate. Unwinding reactions with ssDNA-binding protein contained 5 μ M T7 gp2.5 or 1 μ M of *E. coli* SSB, and these were added with MgCl₂ at the start of the reaction. No DNA trap was added with the MgCl₂ when ssDNA-binding protein was present, but the trap was added with the quenching solution to prevent the unwound strands from re-annealing.

Data analysis. Kinetic fitting was performed using MATLAB with Optimization toolbox software (MathWorks). Stepwise unwinding kinetics were described using the incomplete gamma function^{3,10,25}. Best fits were obtained assuming unwinding by two populations of helicase species with identical step size *s*, but different stepping rates (*k*₁ and *k*₂). The stepping kinetics are described by equation (1):

$$F = A_1 \Gamma(k_1, t, n) + A_2 \Gamma(k_2, t, n) \quad (1)$$

where *F* is the fraction of unwound DNA substrate molecules, *A*₁ and *A*₂ are the amplitudes of unwinding, the incomplete gamma function

$$\Gamma(k, t, n) = \frac{1}{\int_0^\infty e^{-x} x^{n-1} dx} \int_0^{kt} e^{-x} x^{n-1} dx,$$

and *t* is reaction time. The number of steps *n* = (*L* - *L*_m)/*s*, where *s* is step size, *L* is the number of base pairs in the DNA substrate duplex and *L*_m is the length of the shortest DNA duplex that can stay together under the experimental conditions, which was fixed at 12 bp, based on previous studies³.

DNA synthesis kinetics. DNA synthesis kinetics was measured using the rapid quench-flow instrument at 18°C. The final concentrations are reported in parentheses. The DNA substrate with a radiolabelled 34-nucleotide primer (100 nM) was incubated with the DNA polymerase (200 nM) and *E. coli* thioredoxin (2 μ M with T7 DNA polymerase) with or without T7 helicase or Δ Ct T7 helicase (200 nM hexamer) in buffer T containing dTTP (1 mM) and EDTA (1.5 mM). This solution was mixed rapidly with dNTPs (various concentrations) and MgCl₂ (free final Mg²⁺ concentration was 4 mM) in buffer T to initiate the reaction. After pre-determined times, reactions were quenched (200 mM EDTA), resolved by electrophoresis on a 14% or 16% polyacrylamide/7 M urea gel (0.25–0.75-mm wedge spacers), and visualized and quantified using the PhosphorImager (ImageQuant software). The average rate of DNA synthesis was determined from the exponential increase in DNA products with time, *D*_p = *A*(1 - e^{-kt}), where *D*_p is the concentration of DNA products, *A* is the amplitude, *t* is time and *k* is the observed DNA synthesis rate.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature/.

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