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Human Mitochondrial DNA Helicase TWINKLE Is Both an Unwinding and Annealing Helicase*

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**Background:** TWINKLE is the human mitochondrial DNA helicase associated with heritable neuromuscular diseases.

**Results:** TWINKLE has NTPase-dependent DNA unwinding activity and NTPase-independent DNA annealing activity. The unwinding activity is enhanced by displaced strand traps.

**Conclusion:** TWINKLE has more than one ssDNA-binding sites, the one associated with annealing interferes with unwinding in the absence of traps.

**Significance:** The annealing activity may be involved in recombination-mediated replication initiation.

TWINKLE is a nucleus-encoded human mitochondrial (mt)DNA helicase. Point mutations in TWINKLE are associated with heritable neuromuscular diseases characterized by deletions in the mtDNA. To understand the biochemical basis of these diseases, it is important to define the roles of TWINKLE in mtDNA metabolism by studying its enzymatic activities. To this end, we purified native TWINKLE from *Escherichia coli*. The recombinant TWINKLE assembles into hexamers and higher oligomers, and addition of MgUTP stabilizes hexamers over higher oligomers. Probing into the DNA unwinding activity, we discovered that the efficiency of unwinding is greatly enhanced in the presence of a heterologous single strand-binding protein or a single-stranded (ss) DNA that is complementary to the unwound strand. We show that TWINKLE, although a helicase, has an antagonistic activity of annealing two complementary ssDNAs that interferes with unwinding in the absence of gp2.5 or ssDNA trap. Furthermore, only ssDNA and not double-stranded (ds)DNA competitively inhibits the annealing activity, although both DNAs bind with high affinities. This implies that dsDNA binds to a site that is distinct from the ssDNA-binding site that promotes annealing. Fluorescence anisotropy competition binding experiments suggest that TWINKLE has more than one ssDNA-binding sites, and we speculate that a surface-exposed ssDNA-specific site is involved in catalyzing DNA annealing. We propose that the strand annealing activity of TWINKLE may play a role in recombination-mediated replication initiation found in the mitochondria of mammalian brain and heart or in replication fork regression during repair of damaged DNA replication forks.

Reproduction of the human mitochondrial DNA is catalyzed by a minimal replication complex that consists of the helicase (TWINKLE), DNA polymerase (polymerase γA and processivity factor γB), and single strand-binding protein (mtSSB) and strand annealing (2). TWINKLE, encoded at 1q24, was discovered in 2001 (2) and was classified as a helicase based on its sequence similarity (46% amino acid sequence similarity and 15% sequence identity) to bacteriophage T7 gene 4 primase-helicase (T7 gp4) (2, 3). It belongs to the superfamily 4 (SF4) class of helicases, which consists of bifunctional primase-helicase proteins that assemble into ring-shaped hexamers and unwind DNA by the strand exclusion model. In this model, the ring-shaped helicase encircles one of the strands of the duplex DNA and uses energy from nucleotide triphosphate (NTP) hydrolysis to translocate unidirectionally along the single-stranded (ss) DNA and unwind the duplex DNA (4). TWINKLE, similar to phage T7 gp4, contains two domains connected by a linker region. The C-terminal domain has five conserved motifs typical to the SF4 helicases (5). The N-terminal domain contains some of the conserved motifs for the primase activity, but it seems to have lost the primase function (5, 6). Deletion studies have indicated that the N-terminal domain of TWINKLE has retained specific binding activity for single strand (ss) DNA (7). Unlike T7 gp4, TWINKLE can assemble into hexamers and heptamers in the absence of any NTP (5, 8).

Since the discovery of TWINKLE, there have been more than 30 dominant mutations and 3 recessive mutations linked to mitochondrial DNA deletions that manifest in genetically inherited diseases such as progressive external ophthalmoplegia, infantile-onset spinocerebellar ataxia, premature aging, and certain types of cancer (9, 10). Most of the mutations lie in the linker region in TWINKLE, and even though some of the mutant proteins have been preliminarily characterized (3, 6, 11–13), the molecular and biochemical basis of these human diseases are not yet well defined. In addition, all the biochemical activities of TWINKLE related to mitochondrial DNA replication have not been comprehended. This is due to the lack of

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[1] This article contains supplemental Figs. 1–7.

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The peak fractions (600–700 mM NaCl) was analyzed by SDS-PAGE and concentrated using a regenerated cellulose ultrafiltration membrane (cutoff of 30 kDa, Millipore) in an Amicon concentrator. The concentrated fractions were dialyzed against buffer C (50 mM Tris-HCl, pH 7.9, 300 mM NaCl, 50% glycerol), aliquoted, and stored at −80 °C.

DNase contamination of the sample was tested on 72-mer DNA, and nucleic acid contamination was checked by ethidium bromide staining of the sample in a 0.6% agarose gel. The concentration of the final sample was determined spectrophotometrically by measuring $A_{280}$ using the extinction coefficient (ε) 75,000 M$^{-1}$ cm$^{-1}$, 'T7 gp4A' was purified from E. coli as described previously (14, 15).

The Walker A mutant of C-His$_{6}$-TWINKLE was generated by changing the lysine at position 421 to an alanine (K421A mutant) using the QuikChange site-directed mutagenesis XL kit (Stratagene). The presence of the mutation was verified by sequencing the entire gene to ensure that no other secondary mutations apart from K421A were generated.

The mutated clone was transformed into Rosetta BL21 (DE3) placl cells (Novagen). The subsequent steps of cell growth and purification were identical to the purification of wild-type TWINKLE as described above, except that the protein was eluted from the nickel column with the direct addition of 350 mM imidazole instead of a linear gradient, and the heparin-Sepharose column was replaced with a Q-sepharose column (GE Healthcare) for the mutant. The protein was present in the flow-through from the Q column, which was then concentrated using a 30,000 Da molecular weight cutoff membrane in an Amicon concentrator. The protein concentration was determined spectrophotometrically by measuring $A_{280}$ with the extinction coefficient (ε) 75,000 M$^{-1}$ cm$^{-1}$.

Nucleic Acid Substrates—Oligodeoxynucleotides (Table 1) from Integrated DNA Technologies (Coralville, IA) were PAGE-purified, and their concentrations were determined from absorbance at 260 nm. The M13mp18 ssDNA was purchased from United States Biochemical Corp. T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences) were used to radiolabel the DNA, which was subsequently purified by size exclusion chromatography (BioGel P-30 Gel, Bio-Rad). Duplex DNA substrates were generated by mixing complementary strands, heating to 95 °C followed by slow cooling. The 17-bp hairpin loop DNA was made by heating the ssDNA 17hp (Table 1) DNA to 95 °C followed by slow cooling.

Chemical Cross-linking of TWINKLE—Reaction mixtures contained 50 mM triethanolamine-Cl buffer, pH 8.2, 1 mM EDTA, 5 mM diithothreitol (DTT), 1.5 mM NaCl, 5 μM protein (monomer) with or without 4 mM UTP, 10 mM magnesium acetate, 2 μM DNA (GC5040tr + GC5040dis, see Table 1). The protein was cross-linked at 4 °C for defined times using freshly prepared dimethyl suberimidate (Thermo-Scientific) (DMS, 10 mg/ml) from a 50 mg/ml stock in ice-cold triethanolamine- HCl with pH 8.2 adjusted by 3 N NaOH. The reaction was stopped by the addition of an equal volume of 1 M glycine and analyzed by 4–12% BisTris SDS-PAGE (Invitrogen). The protein bands were detected by Coomassie Brilliant Blue (R-250) staining.
DNA Binding Measured by Fluorescence Anisotropy Titration—Fluorescence anisotropy was measured (16) on a Fluoromax-4 spectrofluorometer (Horiba Scientific, NJ) mounted with a thermoelectrically controlled cell holder. Concentrated TWINKLE was added to 5 mM tetramethylrhodamine (TMR)-labeled ssDNA (5’-TMRtr, see Table 1) or TMR-labeled dsDNA (TMR-labeled 5’-TMRtr annealed to 5’-TMRdis, see Table 1) in a reaction buffer containing 50 mM Tris acetate, pH 7.5, 5 mM magnesium acetate, 1 mM EDTA in the absence of magnesium acetate, 5 mM DTT, annealed to 5’-TMRdis, see Table 1) in a reaction buffer containing 50 mM Tris acetate, pH 7.5, 1 mM EDTA, TWINKLE hexamer (100 nM), and indicated amounts of DNA (ss, ds, or M13) and UTP mixed with magnesium acetate (6 mM excess magnesium acetate than total concentrations; 40 mM ssDNA trap or 20 nM T7 gp2.5 where indicated). The reactions were incubated at 30 °C for indicated times and stopped with 12 reaction volumes of quenching solution (100 mM EDTA, pH 8.0, 1% SDS, 0.5% bromphenol blue). The amount of dsDNA and unwound ssDNA was quantified from 15% nondenaturing polyacrylamide/TBE gel using PhosphoImager and ImageQuant (GE Healthcare) and fitted using Sigma Plot software. The fraction of ssDNA generated was calculated from Equation 4,

\[
F = \frac{(SS \times DS_0) - (DS \times SS_0)}{(DS_0 \times (SS + DS))}
\]  

where \(F\) is the fraction of unwound substrate, \(DS\) and \(SS\) are intensities of duplex and unwound substrate bands at a given time, respectively, and \(DS_0\) and \(SS_0\) are intensities of duplex and unwound substrates at time 0, respectively. The fraction of ssDNA against time was fit to the exponential Equation 5,

\[
F = A \cdot (1 - e^{-bt})
\]

where \(A\) is the maximum fraction of ssDNA that can be generated enzymatically from the substrates; \(b\) is the first-order rate constant of DNA unwinding; \(t\) is reaction time. If the reactions did not reach completion in the time scale of measurement, the data were fit to a straight line (Equation 6),

\[
F = yo + b \cdot t
\]

The S.E. were obtained from data fitting in the Sigma Plot software, and the initial rate was determined by multiplying the amplitude with the corresponding rate, and the S.E. for the initial rate was calculated by combining S.E. of both the rate and the amplitude using Equation 7.

\[
S.E.(initial\ rate) = (rate \times S.E._{amp}^2 + Amp \times S.E._{rate}^2)^{1/2}
\]

DNA Annealing—The DNA annealing reaction was carried out at 30 °C using the indicated amounts of ssDNA and TWINKLE for defined times in reaction buffer containing 50 mM Tris acetate, pH 7.5, 0.01% Tween 20, 5 mM dithiothreitol, 1 mM EDTA with or without 4 mM UTP, and 6 mM free magnesium acetate. Samples were immediately loaded on a nondenaturing polyacrylamide/TBE gel running at 60 V, after quenching with 12× reaction volume of the quenching solution. The amount of annealed DNA and ssDNA was quantified using PhosphoImager and ImageQuant (GE Healthcare) and fit to Equation 4 (by replacing \(SS_0\) with \(DS_0\) and vice versa) using Sigma Plot software to obtain the rate constant and amplitude of annealing.

Annealing with prebound hairpin DNA (17hp), 20 base dsDNA (5’-TMRtr + 5’-TMRdis without the TAMRA label), or dT100 ssDNA was performed by incubating increasing

\[
F = \frac{(SS \times DS_0) - (DS \times SS_0)}{(DS_0 \times (SS + DS))}
\]
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concentrations of the above DNAs with 10 nM TWINKLE (hexamer) for 15 min at 30 °C followed by addition of 2 nM of the two ssDNAs sequentially complementary to each other with 4 mM UTP and 6 mM free magnesium acetate. Reactions were done at 30 °C for 2 min. Samples were immediately loaded on a 4–20% nondenaturing polyacrylamide gel running at 60 V, after quenching with 5× reaction volumes of the quenching solution.

All protein concentrations mentioned signify hexameric concentration. For the unwinding and annealing assays, the protein was preincubated with DNA in the presence of UTP, and the reaction was started with magnesium acetate. Re-annealing traps for unwinding assay (where mentioned) were added with magnesium acetate. For UTPase assay, the reactions were started by addition of the protein.

RESULTS

Expression and Purification of C-His6-TWINKLE from E. coli—The expression of TWINKLE in E. coli allowed for easy and large scale purification of the homogeneous protein. We estimate that our purification protocol yields 20–30 mg of pure C-His6-TWINKLE per 100 g of cells, which is comparable with the yield using the insect cell system (5). The C-His6-TWINKLE was expressed at low levels in E. coli as visualized after IPTG induction (Fig. 1A, boxed area, lane 5) when compared with the significant amount of expression of the N-His6-TWINKLE (Fig. 1A, boxed area, lane 3). Curiously, the N-His6-TWINKLE protein was insoluble under varied induction temperature conditions, whereas the C-His6-TWINKLE was soluble. TWINKLE resolved by SDS-PAGE was identified by proteolytic digestion followed by nano-LC MS/MS. Using mass spectrometry, we identified a closely associated band as GroEL eluting with TWINKLE from the Ni2+-Sepharose 6 Fast Flow column. A substantial amount of TWINKLE was therefore sacrificed to obtain pure protein with a minimum amount of GroEL (Fig. 1A, lane 7). After chromatography through the heparin-Sepharose column, the protein was ~90% pure (Fig. 1A, lanes 8 and 9). We observed that 300 mM NaCl and DTT were required for protein solubility and stability, which was further enhanced in the presence of 50% glycerol (Fig. 1A, lane 10). There was no observed DNase contamination in the final sample (Fig. 1B, lanes 5–7). A recent study has reported the purification of TWINKLE from E. coli using a clone in pET21a vector with C-His6 tag preceded by AALEE after the gene sequence. Although the clone was similar to the one we used in this study (pTriEx vector with C-His6 tag preceded by RPPRP after the gene sequence), the purification protocol required the use of Mg2⁺ and ATP through all the steps to keep TWINKLE soluble (17). However, our protocol provides soluble TWINKLE without added Mg2⁺ and ATP, and hence the final protein sample is free of MgATP, which allows in vitro studies related to the effect of these cofactors on the biochemical properties of TWINKLE.

E. coli Purified TWINKLE Forms Oligomers Spontaneously but Specifically Forms Hexamers in the Presence of MgUTP—To characterize the oligomeric state of the recombinant TWINKLE purified from E. coli, we performed DMS cross-linking of the protein in the presence and absence of cofactors (Mg2⁺ and UTP). DMS is a homobifunctional cross-linking reagent that can cross-link surface lysines within 11 Å of each other (18). In the absence of the cross-linking reagent, we observed some dimers (Fig. 2A, lanes 6 and 12) consistent with a previous study where ATPγS was used (8). In the presence of the cross-linking reagent DMS, but in the absence of Mg2⁺ and UTP, we were able to capture TWINKLE oligomers (Fig. 2A, lanes 7–11). However, when Mg2⁺ and UTP were present, hexamers accumulated to a greater level as opposed to the higher oligomers (Fig. 2A, lanes 1–5). Our cross-linking results show that Mg2⁺ and UTP stabilize the hexameric state of TWINKLE. Although dsDNA was present in both experiments, we show that in the presence and absence of the DNA TWINKLE possesses similar oligomeric states (Fig. 2B, lanes 1 and 2). Thus, similar to the protein purified from the insect cells (8), E. coli expressed TWINKLE forms hexamers and higher oligomers in the presence and absence of MgUTP. However, we show that hexamers are stabilized selectively in the presence of MgUTP.
TWINKLE Binds ssDNA and dsDNA Fragments in the Absence of Cofactors.—The DNA binding properties of TWINKLE were explored using fluorescence anisotropy titrations, where TMR-labeled ssDNA or dsDNA (attached to the 5'-end) was titrated with increasing TWINKLE concentrations. The titration data clearly show that TWINKLE binds both ssDNA and dsDNA with or without Mg$^{2+}$ or nucleotides (UTP) (Fig. 3, A and B). The equilibrium measurement provided the $K_d$ of TWINKLE complexed with ssDNA as 3.2 ± 2.8 nM without cofactors, 5.4 ± 2.4 nM in the presence of Mg$^{2+}$, and 4.4 ± 2.6 nM in the presence of Mg$^{2+}$ and an unhydrolyzable analog of UTP, UMPPNP (Fig. 3A). TWINKLE binds dsDNA about 2-fold more tightly ($K_d$ is 1.2 ± 0.3 nM without cofactors, 2.1 ± 0.4 nM with Mg$^{2+}$, and 2.6 ± 1.4 nM with Mg-UMPPNP) (Fig. 3B). This is unlike T7 gp4 helicase, which binds preferentially to ssDNA over dsDNA, and moreover it has a strict requirement of dTTP or dTTP analog to bind ssDNA (19, 20). Thus, our results are consistent with previous reports that indicate that TWINKLE binds to ssDNA (8) and dsDNA (21) without MgUTP, and our equilibrium $K_d$ measurements show that TWINKLE binds to these DNAs very tightly with a preference for binding dsDNA.

The helicase activity of TWINKLE was measured by monitoring the unwinding of a short radiolabeled ssDNA annealed to M13 ssDNA. Two 60-nucleotide short ssDNAs (M1320tr5’T or M1320tr3’T, see Table 1) were so prepared that they formed a 20-bp duplex region on the M13 ssDNA, whereas the remaining 40-nucleotide region formed either a 5'-tail or a 3'-tail, referred to as the 5'-tailed substrate or the 3'-tailed substrate, respectively. The 20-bp dsDNA length was chosen based on the report that TWINKLE can unwind only short stretches of dsDNA (1). The unwinding reactions were carried out by pre-incubating 25 nM TWINKLE with the unwinding substrate (0.4 nM) in the presence of various NTPs (4 mM) and initiating the reactions with Mg$^{2+}$. Control experiments in the absence of TWINKLE or NTP showed no unwinding (supplemental Fig. 1, A and B), indicating that the 20-bp duplex is not spontaneously unwound under our assay conditions and that unwinding is contingent upon NTP hydrolysis-dependent activity of TWINKLE. Additionally, the Walker A mutant K421A of TWINKLE failed to unwind the same DNA with 5'-tail even at higher hexamer concentrations (supplemental Fig. 1C). This demonstrates that the observed NTP-dependent DNA unwinding is due to the activity of TWINKLE and not any other contaminating helicases in the protein sample. Comparison of the initial rates of unwinding (Fig. 4A) of the 5'-tailed substrate revealed most efficient unwinding in the presence of dATP, with slightly lower but comparable rates in the presence of UTP, ATP, and dGTP. The initial rate of unwinding supported by GTP was half of UTP, ATP, and dGTP, whereas dCTP, CTP, and dTTP supported very little unwinding (Fig. 4A and supplemental Fig. 2).

In contrast to the unwinding of the 5'-tailed substrate, the unwinding of the 3'-tailed substrate was not completed and was...
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3–6 times slower under the same conditions (Fig. 4B). Nonetheless, it was clear that UTP and ATP supported more efficient unwinding and dTTP or CTP did not support any unwinding (Fig. 4B and supplemental Fig. 3). The significantly lower fraction of DNA unwound with the 3′/H11032-tailed substrate compared with the 5′/H11032-tailed substrate has been recently reported (22). This suggests that TWINKLE favorably binds to the end of the oligonucleotide with the 5′/H11032-tail than to the circular ss region of the M13 ssDNA, probably because the free end of the 5′/H11032-tail allows threading of the hexameric helicase, as opposed to the circular M13 ssDNA region where the hexamer has to open and assemble around the DNA. This is a strikingly different behavior from T7 gp4 helicase, which preferentially loads on to the long ss region of the M13 ssDNA and therefore unwinds the 3′-tailed substrate more efficiently than the 5′-tailed substrate (22, 23).

We measured the UTP hydrolysis $V_{\text{max}}$ and $K_m$ values under similar conditions of unwinding both in the presence and absence of various types of DNA and at varied UTP concentrations. UTP hydrolysis was only marginally stimulated by 25 nM M13mp18 ssDNA or by 1 μM ds fork DNA over no DNA (Table 2). The $K_m$ value of UTP in the absence of DNA was 3.4 mM, which is ~2 times higher than in the presence of M13 ssDNA (1.7 mM). The $K_m$ value for ATP in the presence of activated calf thymus DNA has been reported to be 1.4 μM (17), which is similar to what we found for UTP with all DNAs.

**TABLE 2** Determination of $K_m$ and $V_{\text{max}}$ values of TWINKLE with UTP using different DNA substrates

<table>
<thead>
<tr>
<th>DNA</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (μM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssM13mp18</td>
<td>1.7</td>
<td>15.6</td>
</tr>
<tr>
<td>ssDNA</td>
<td>1.6</td>
<td>12.8</td>
</tr>
<tr>
<td>Forked dsDNA</td>
<td>1.4</td>
<td>11.2</td>
</tr>
<tr>
<td>No DNA</td>
<td>3.4</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Unwinding Activity of TWINKLE Is Facilitated by Trapping of the Displaced Strand—Further analysis of the DNA helicase activity of TWINKLE revealed that the initial unwinding rate of the 5′-tailed substrate was 7-fold faster when we added T7 gp2.5 single strand-binding protein during the addition of Mg$_2^+$ (Fig. 5A). It has been shown that mtSSB stimulates unwinding by TWINKLE by almost 8-fold in a helicase assay similar to the M13-based unwinding assay that we have used here (24). Because the heterologous T7 gp2.5 stimulates the helicase activity of TWINKLE, it appears that the stimulation by single strand-binding proteins is not protein-specific. T7 gp2.5 by itself does not unwind dsDNA in the absence of TWINKLE (Fig. 5A). This is unlike E. coli SSB, human replica-
tion protein A, and T7 gp32 single strand-binding proteins, which are in many ways similar to mtSSB. These SSB proteins have been shown to capture thermally melted bases to effectively unwind dsDNA (25). One mechanism by which the single strand-binding proteins can increase the helicase activity is by coating the M13mp18 single strand and preventing TWINKLE from getting trapped by the extensive ssDNA region. In this manner, the SSBs can have a nonspecific role in increasing the free TWINKLE concentration for its effective encounter with the 5''/H11032T tail of the ssDNA annealed to the circular M13 ssDNA. In addition, the SSB protein can increase the efficiency of DNA unwinding by preventing re-annealing of the newly unwound displaced strand. Consistent with the latter role, we observed increased unwinding when unlabeled M1320tr5''T strand was added at the start of the reaction with the Mg^{2+} (Fig. 5A, left-most panel). The unlabeled M1320tr5''T can anneal to M13 ssDNA and hence acts as a displaced strand trap to prevent re-annealing of the radiolabeled M1320tr5''T back to the M13 ssDNA circle. The displaced strand trap and gp2.5 were essential for unwinding longer DNA tracts of 55 bp by TWINKLE (Fig. 5B). Similarly, TWINKLE could unwind the 3''-tailed M13 partial duplex more efficiently in the presence of gp2.5 and the displaced strand trap than in the absence (Fig. 5C).

**TWINKLE Possesses DNA Strand Annealing Activity**—The finding that unwinding is greatly aided by trapping of the newly unwound displaced strand prompted us to test if TWINKLE has the ability to promote annealing of complementary strands.

![FIGURE 5. Effect of single strand-binding protein and the reannealing DNA trap on the unwinding kinetics by TWINKLE. A, 5''-tailed substrate was unwound in the presence of 25 nM TWINKLE and 4 mM UTP in the presence of 40 nm unlabeled ssDNA displaced strand trap or 2 μM T7 gp2.5. The rates of unwinding and fraction of unwound ssDNA product with the corresponding S.E. under four different conditions tested in the unwinding assay are summarized in the table. A plot of fraction of DNA unwound over time in the presence of T7 gp2.5 trap (square) and ssDNA trap (triangle), in the absence of trap (circle), and in the presence of T7 gp2.5 trap but absence of TWINKLE (diamond) is also shown. The reaction times are 2, 5, 15, 30, 60, and 90 min. B, unwinding of the longer 55-bp M13ds DNA is better in the presence of DNA trap and gp2.5 than in their absence. The unwinding reaction was carried out under the same conditions as the 20-bp M13ds DNA except that the trap DNA is the 40T55-bp in place of the 40T20-bp DNA. C, plot of fraction of 3''-tailed substrate DNA unwound over time in the presence of 2 μM T7 gp2.5 trap (square), 40 nm ssDNA displaced strand trap (triangle), and in the absence of trap (circle).
ssDNAs. We tested the annealing of two ssDNA oligonucleotides (GC5040tr and GC5040dis, Table 1) capable of forming a 40-bp duplex region in a fork in the presence and absence of TWINKLE (Fig. 6A). The two ssDNAs at 2 nM concentration formed a duplex within 2 min in the presence of TWINKLE, whereas in the absence of TWINKLE, there was negligible annealing observed (Fig. 6B). T7 gp4 helicase under similar conditions did not exhibit any annealing activity of the partially complementary (GC5040tr and GC5040dis, Table 1) or of the completely complementary DNAs (Blunt 20 or 58tr and to Blunt 20 58dis, see Table 1) (supplemental Fig. 4).

To better understand the mechanism of TWINKLE-mediated DNA strand annealing, we first tested the effect of UTP and Mg$^{2+}$/H$^{11001}$ on its strand annealing activity. TWINKLE-mediated DNA annealing was observed under all conditions, and the yields of the annealed products or the amplitudes of the reaction were comparable, although the initial rate of annealing was almost two times faster in the presence of UTP and Mg$^{2+}$/H$^{11001}$ than in its absence (Fig. 6C and D). TWINKLE could anneal the two DNA strands even when UTP was replaced by its nonhydrolysable analog UMPPNP (supplemental Fig. 5).

We also tested the effect of TWINKLE concentration and NaCl concentration on the strand annealing activity. The annealing rate did not show a significant change from 6 to 100 nM TWINKLE hexamer concentration (Fig. 6E). However, the annealing reaction was sensitive to NaCl concentration and showed a drastic decrease in a narrow range between 50 and 125 mM NaCl concentration, and very little annealing was observed beyond 150 mM NaCl (Fig. 6F and G). The salt dependence is consistent with an active role of TWINKLE in

FIGURE 6. DNA strand annealing activity of TWINKLE. A, schematic representation of two partially complementary ssDNAs annealing to form a 40-bp forked DNA. B, annealing assay was performed by mixing 2 nM ssDNA (GC5040tr + GC5040dis, see Table 1) at 30 °C in the presence and in the absence of 50 nM TWINKLE and 4 mM MgUTP for 1, 5, 10, 15, and 30 min. C, 18% acrylamide/TBE gels show the effect of Mg$^{2+}$/H$^{11001}$ and UTP on the annealing of the 40-bp forked DNA at 30 °C in the presence of 10 nM TWINKLE and 2 nM DNA for 0, 15, 30, 60, 120, 300, and 600 s. D, amount of annealed product formed is normalized to the amount of product at time 0 and plotted against time and fit to Equation 2 to get the rate and amplitude. The initial annealing rate (fraction annealed/min) in the absence of MgUTP is 0.02 ± 0.002, in the presence of magnesium acetate is 0.03 ± 0.004, in the presence of UTP is 0.01 ± 0.001, and in the presence of MgUTP is 0.04 ± 0.003. E, fraction of annealed product in 2 min at varying TWINKLE hexamer concentrations (6, 12.5, 25, 50, and 100 nM). F, 12% acrylamide/TBE gel shows annealing of 2 nM DNA by 10 nM TWINKLE at 30 °C in 2 min at varying NaCl concentrations (0, 10, 50, 100, 110, 125, 150, 175, 200, 300, 400, and 500 mM). The prequenched controls for each salt concentration are shown in the lanes marked 0 under each salt concentration, and the 2-min reaction for each salt concentration is marked as 2. G, amount of annealed product formed is normalized to the product formed at respective 0 and plotted against salt concentration.
DNA annealing. We expect spontaneous DNA annealing to show an opposite behavior, i.e. base-pairing is more favorable at high concentrations of NaCl as base-stacking interactions are strengthened with an increase in salt concentration (26). It is known that TWINKLE-DNA interactions are salt-sensitive and are lost at 420 mM NaCl (17). The results indicate a role of the DNA-binding site of TWINKLE in the observed DNA strand annealing activity.

We next assessed the dependence of annealing rate and amplitude on the length of the DNA to be annealed. We used 0.2 nM complementary ssDNAs of 20, 40, and 58 bases (Blunt 20, 40, or 58tr and Blunt 20, 40, or 58dis). The short 20-base DNA did not show a clear annealed product but instead a smeary band moving up with increasing reaction times (supplemental Fig. 6). However, both the 40- and 58-base DNAs efficiently annealed in the presence of TWINKLE with similar amplitudes, comparable with the forked DNA (supplemental Fig. 6). Our fluorescence anisotropy-based binding assays above show that a 20-base ssDNA binds tightly to TWINKLE. Therefore, the lower efficiency of the short DNA annealing could be because the DNA-binding site of TWINKLE protects the entire 20-base DNA, and thus most included bases within the binding site of the TWINKLE are not available for base-pairing. The longer 40- or 58-base ssDNAs must have exposed bases that can pair with the complementary ssDNA easily. Interestingly, if T7 gp2.5 was prebound to one of the two strands, strand annealing by TWINKLE was inhibited as expected. The partial inhibition could be because TWINKLE may be able to dislodge T7 gp2.5 (supplemental Fig. 7).

**TWINKLE-mediated Strand Annealing Is Inhibited by ssDNA but Not by dsDNA**—Our DNA binding studies (Fig. 3) showed that ssDNA and dsDNA bind TWINKLE with comparable $K_d$ values. To determine the role of the ssDNA- and dsDNA-binding sites of TWINKLE in strand annealing, we carried out annealing reactions in the presence of increasing amounts of ssDNA or dsDNA competitors (10–50 nM), which were preincubated with TWINKLE before the addition of the annealing substrates and MgUTP. Preincubation of TWINKLE with increasing amounts of ssDNA competitor inhibited the TWINKLE-mediated annealing reaction (Fig. 7A). Interestingly, preincubation of TWINKLE with increasing amounts of the dsDNA competitor, which binds very tightly as shown in Fig. 3A, had no effect on the annealing reaction (Fig. 7A). The distinct effects of ssDNA and dsDNA on the annealing activity of TWINKLE suggest more than one DNA-binding site on TWINKLE with distinct affinities for ssDNA and dsDNA, and only the ssDNA-specific site appears to promote the strand annealing reaction.

It is proposed that the homologous T7 gp4 hexamer binds ssDNA and dsDNA in its central channel (27), but the N-terminal primase domains are also capable of binding ssDNA (4). It is very likely that TWINKLE similar to T7 gp4 binds ssDNA and dsDNA in the central channel. In addition, as suggested by a previous report (3), the N-terminal domains of TWINKLE may specifically bind ssDNA, and these surface-exposed site(s) may be involved in catalyzing the DNA annealing reaction.

To directly determine whether ssDNA has more than one binding site on TWINKLE, we compared the $K_d$ value of ssDNA (Fig. 3A) on TWINKLE and the $K_d$ value of ssDNA against pre-bound 20-bp dsDNA on TWINKLE using fluorescence anisotropy-based competition assay. We incubated 7 nM TWINKLE with 6 nM TMR-labeled RD-ds20 and 7 nM TWINKLE was titrated with increasing ssDNAs ($\text{dT}_{100}$, TMRtr) to obtain the ssDNA IC$_{50}$ of 672 nM and $K_i$ of 174 nM.

**FIGURE 7.** Competition between dsDNA and ssDNA in binding and in TWINKLE-catalyzed strand annealing reaction. A. 12% acrylamide/TBE gels show the annealing of ssDNA to form a 40-bp forked dsDNA in a reaction at 30 °C in the presence of 10 nM TWINKLE, 2 nM DNA, 4 mM UTP, and 6 mM free magnesium acetate conducted for 2 min. In these reactions, TWINKLE was preincubated with increasing concentrations of competitors such as ssDNA (dT100) or dsDNA (17-bp hairpin) for 15 min before adding the ssDNAs to be annealed. B, complex of 6 nM TMR-labeled RD-ds20 and 7 nM TWINKLE was titrated with increasing ssDNAs (5'-TMRtr). The fluorescence anisotropy decrease was fit to Equations 2 and 3 to obtain the ssDNA IC$_{50}$ of 672 nM and $K_i$ of 174 nM. C, model showing potential ssDNA-binding modes by TWINKLE.
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ssDNA led to an increase in anisotropy at first and then a decrease with addition of higher amounts of ssDNA (Fig. 7B). The small increase in anisotropy could be due to ssDNA binding to TWINKLE. The decrease in anisotropy due to unbinding of dsDNA was fit to a competitive binding model, which provided ssDNA $K_i$ of $\sim 174$ nM. This $K_i$ value is much larger than the measured ssDNA $K_i$ of $3–5$ nM (Fig. 3A). This result supports two types of ssDNA-binding sites on TWINKLE. We propose that TWINKLE can bind ssDNA both in the central channel and to a surface-exposed site(s) (Fig. 7C).

**TWINKLE Actively Recruits ssDNA Trap for Complementary Base-pairing with the Displaced Strand during Unwinding**—The surface-exposed sites proposed to be involved in the strand annealing reaction are likely to be involved in binding the displaced strand trap during DNA unwinding. If the trap is pre-bound to TWINKLE, it can effectively base pair with the newly unwound DNA to stimulate the helicase activity of TWINKLE. To test this hypothesis, we designed the following experiments: DNA unwinding of the 55-bp DNA was measured in the presence of the displaced strand trap and in the presence of a non-complementary dT100 homopolymer together with the displaced strand trap. TWINKLE was mixed with the M13-based forked DNA substrate prior to addition of the DNA traps. We observed that unwinding was efficient in the presence of the displaced strand trap (Fig. 8A) consistent with the above results shown in Fig. 5. Interestingly, unwinding was poor when dT100 was present with the displaced strand trap (Fig. 8A).

We propose that dT100 (present in excess of the displaced strand trap) binds to the surface-exposed ssDNA-binding sites on TWINKLE and prevents the displaced strand trap from binding (Fig. 8B). Thus, the only way the displaced strand trap can base pair with the newly unwound DNA is after binding from solution, which is an inefficient process at the low concentration of displaced strand trap used in our studies (40 nM). Thus, the results are consistent with the model that TWINKLE binds ssDNA on a surface-exposed site(s), and if the ssDNA happens to be complementary to the unwound DNA, then it stimulates DNA unwinding by trapping the newly unwound DNA strand.

**DISCUSSION**

In this paper, we report a purification protocol to obtain soluble and stable TWINKLE from *E. coli* without any co-factor contaminants, and we demonstrate for the first time the DNA strand annealing activity of TWINKLE. Our results also suggest that TWINKLE has distinct duplex and ssDNA-binding sites, and ssDNA can bind to multiple sites outside of the central channel.

A recently reported purification protocol for bacterially expressed TWINKLE used Mg$^{2+}$ and ATP in the protein preparation buffers to keep the protein soluble, and these cofactors remained through the final steps (17). We show here that TWINKLE can be obtained in soluble form without any cofactor contaminants, which will be important for future structural studies as well as to study the effect of cofactors on the properties of the protein. We observed, as with the insect cell purified TWINKLE, the *E. coli* expressed protein is more stable at a high salt concentration (>300 mM NaCl). The oligomerization properties and the NTP dependence of the unwinding activity of our protein demonstrate that irrespective of the source of protein, the fundamental biochemical properties of TWINKLE are the same.

In our protein-protein cross-linking study, oligomers were only identifiable at a high salt concentration (1.5 M) possibly because protein aggregation at the lower salt concentrations leads to the formation of multimers that cannot be resolved on a polyacrylamide gel. The protein-protein cross-linking studies revealed that even though TWINKLE forms both hexamers and heptamers, the hexamers are enriched in the presence of MgUTP. The existence of both hexameric and heptameric structures is known among ring-shaped helicases such as the T7 gp4 and *Methanothermobacter thermoautotrophicus* minichromosome maintenance with the existence of double-hexamer or double-heptamer in case of the latter (28, 29). In T7 gp4, heptamers are predominantly present with dTDP, and these species apparently do not bind DNA (29). It has been postulated that the helicase ring opens after the DNA contacts the N-terminal primase domain, and during this process one of the subunits of the heptamer is lost (29–31). Perhaps, in the presence of MgUTP, the preformed TWINKLE heptamer loses...
one of its subunits to form an open hexameric ring that encircles the DNA. Although TWINKLE has been demonstrated to unwind short DNA duplexes with a directionality of 5’ to 3’ on the strand it translocates, its helicase activity has not been characterized in detail. In this study, we designed our helicase assays based on the unwinding properties of the T7 gp4, where it is known that on an M13 ssDNA-based assay, the helicase assemblies on the M13 ssDNA and moves in the 5’ to 3’ direction to unwind the short duplex with a 3’-tail (23, 32). We, however, observed that TWINKLE does not unwind the 3’-tail DNA substrate very efficiently; instead it unwinds the 5’-tail substrate efficiently. This result indicates that TWINKLE can load more easily on the 5’-tail than the 3’-tail DNA substrate, and if it is bound to the DNA ssDNA. This raises an obvious question as to how TWINKLE loads on DNA in the cell, where linear 5’-tail-like structures are not common. Most of the known ring-shaped helicases have an assigned loader that helps them to assemble around the DNA (33). Because TWINKLE can form a ring in the absence of cofactors or DNA, it might need a loader to open the ring and load on circular DNA. Thus, lack of a loader in vitro limits loading of TWINKLE on DNA, leading to slow unwinding rate by TWINKLE as compared with T7 gp4. When this manuscript was in preparation, a study on TWINKLE demonstrated that TWINKLE can load on a closed circular ssDNA molecule to initiate unwinding without the requirement of a specific loader (22). In concurrence with our results, the same study showed that unwinding efficiency with the M13-DNA-based assay was more with the 5’-tail substrate than with the 3’-tail substrate under all the conditions tested. Taken together, this indicates that although TWINKLE can load on a circular ssDNA unaided by a separate loader, the loading efficiency might be significantly higher on an ss tail having a free end than on an ss closed circle. Therefore, we cannot exclude the possibility of the requirement of a loader by TWINKLE during initiation of replication in vivo.

Additionally, we observed that TWINKLE unwinds the DNA more efficiently in the presence of the displaced strand DNA trap or single strand-binding protein. This is observed in some helicases of the RECQ family, e.g. forked dsDNAs are unwound by RECQ5β only in the presence of the human replication protein A and by RECQ4 only in the presence of the displaced strand DNA trap (34, 35). It was shown that both RECQ4 and RECQ5β possess strand annealing activity, which therefore masks their unwinding activity (34, 35). This led us to test and confirm that TWINKLE can anneal two complementary ssDNAs efficiently.

We find that TWINKLE can efficiently anneal two complementary ssDNA with and without Mg2+ and NTP. As of now, the DNA binding properties of the TWINKLE are not well understood. Previous protein-DNA gel-shift assays indicated that TWINKLE binds ssDNA only in the presence of the cofactors (3). However, recent studies by limited proteolysis of TWINKLE have indicated that it binds ssDNA in the absence of cofactors, although its structural conformation differs in their presence (8). Our fluorescence anisotropy-based DNA binding studies show that TWINKLE can bind DNA in the absence of cofactors (Fig. 3). Because TWINKLE can bind ssDNA in the absence of the cofactors, it can catalyze the strand-annealing reaction even without MgUTP. We observed that high salt (>150 mM NaCl) inhibits the strand annealing activity of TWINKLE. At high salt, the protein-DNA interactions are disrupted (26), which leads to a lowering of the strand annealing efficiency. The length of the ssDNA to be annealed also affects the efficiency of strand annealing, and we observed little annealing of the 20-base DNAs. One explanation is that the bound protein occludes a part of the ssDNA; therefore, the longer 40- and 58-base DNAs are annealed with a higher efficiency in the presence of TWINKLE.

The cofactor independent strand annealing activity must depend on TWINKLE bringing together two complementary ssDNAs. Based on our results, a number of mechanistic models can be conceived for the strand annealing activity by TWINKLE. A single ring may bind two ssDNA molecules, and if they are complementary and brought in close proximity by TWINKLE, base-pairing occurs at a faster rate than by simple protein independent collision. Alternatively, two separate rings, each bound to one ssDNA, can closely interact in space bringing the complementary ssDNA together for base-pairing. We have eliminated this second model based on the fact that an increase in protein concentration from 6 to 100 nm does not significantly change the annealing efficiency (Fig. 7E). A third possibility is that TWINKLE may bind ssDNA in such a way that its conformation is conducive for facile base-pairing with a complementary DNA from solution. The ssDNA binding to the N-terminal domain represents an outer surface that may provide such a site for DNA annealing. However, further experiments are needed to test this model.

Strand annealing activity is observed in many helicases (34–37), but its biological role has not been defined precisely. It might be helpful in recombination-mediated replication initiation, which has been proposed to occur in mammalian heart and brain mitochondria (38), or in replication fork regression during the repair of damaged DNA replication forks (36). Therefore, chances are that TWINKLE-mediated strand annealing plays a role in similar processes in mitochondria; however, additional studies with the rest of the components of the replication machinery such as mtSSB and DNA polymerase γAB are needed to establish such roles of the TWINKLE.

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