

DNA and RNA–DNA annealing activity associated with the τ subunit of the *Escherichia coli* DNA polymerase III holoenzyme

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ABSTRACT

The DNA polymerase III (pol III) holoenzyme is the 10 subunit replicase of *Escherichia coli*. The 71 kDa τ subunit, encoded by *dnaX*, dimerizes the core polymerase ($\alpha\epsilon\theta$) to form pol III' [$(\alpha\epsilon\theta)_2\tau_2$]. τ is also a single-stranded DNA-dependent ATPase and can substitute for the γ subunit during initiation complex formation. We show here that τ also possesses a DNA–DNA and RNA–DNA annealing activity that is stimulated by Mg^{2+} , but neither requires ATP nor is inhibited by non-hydrolyzable ATP analogs. This suggests the τ may act to stabilize the primer–template interaction during DNA replication.

INTRODUCTION

The DNA polymerase III holoenzyme (pol III HE) is the replicase of *Escherichia coli* (1). It is composed of 10 subunits that assort in the following manner: α , ϵ and θ form the core (2) that possesses both polymerase function (in α) (3) and the 3'→5' proofreading exonuclease (in ϵ) (4). The addition of τ dimerizes the core to form pol III' (5), a slightly more processive polymerase than the core (6,7). γ , δ , δ' , χ and ψ form the γ complex (8) that acts to place β , the processivity factor (9), onto the primer–template. The addition of the γ complex to pol III' forms pol III* (10). Pol III HE is completed by the addition of β to pol III* (8,10).

Pol III HE is extraordinarily processive. It is likely to synthesize a leading strand of 2.2 Mb *in vivo* in a single primer binding event. When incorporated into a replication fork *in vitro*, processivities in the range of 0.5 Mb have been measured (11,12). This processivity arises from the ring-like structure of the β dimer (13), acting to clamp the core onto the template.

τ is encoded by *dnaX* (14,15) and is a single-stranded DNA-dependent ATPase (16). τ can substitute for γ , which is also encoded by *dnaX* (15,17) and arises as a result of a translational frame-shift (18–20), in loading the β dimer onto DNA (21). γ , on the other hand, cannot dimerize the core (22). Recent studies using replication forks reconstituted with pol III HE and the ϕ X-type primosomal proteins have suggested that τ was required

for rapid cycling of the lagging strand core from the 3'-end of the just-completed Okazaki fragment to the 3'-end of the new primer for the next Okazaki fragment (23).

The recent cloning of the genes encoding the δ , δ' , χ , ψ and θ subunits and the overexpression of their gene products (24–31) now allows a detailed investigation of the role of each pol III HE subunit at the replication fork. In the course of our investigations we have found that τ possesses both a DNA–DNA and an RNA–DNA annealing activity. Even though τ has DNA-dependent ATPase activity, neither ATP nor AMPPNP had any effect on the annealing reaction.

MATERIALS AND METHODS

Enzymes and reagents

DnaG and the *E. coli* single-stranded DNA binding protein (SSB) were as described (32,33). Purified subunits of the DNA pol III HE (core, β , τ , δ , δ' , χ and ψ) were the gift of Drs G. Dallmann, D. R. Kim, M. Olson and C. S. McHenry (University of Colorado Medical Center). ATP, ATP analogs and T4 polynucleotidyl kinase were from Pharmacia LKB Biotechnology Inc. [α - 32 P]dATP and [γ - 32 P]ATP were from Amersham. The large fragment of DNA polymerase I (Klenow fragment) was from Boehringer Mannheim. pBR322 *MspI* digests were from New England Biolabs. M13Gori1 DNA was prepared as described (34).

DNA and RNA substrates

Two pairs of complementary oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. The 78mer top strand had the sequence 5'-GAT CTA GAT ATC TCT GGCGGTGTTGACGCGTAGATCTTAATACGACT CAC TAGACGCGTACCACTGGCGGTGATAT-3', whereas the sequence of the 78mer bottom strand was the complement. The DNA sequence of the 30mer was 5'-CAAAAAGGGCGCTATATCCACTCCACCGA-3', whereas the RNA sequence of the 30mer was 5'-UCG GUG GAG UGG AUA UAG CGC CCU UUUUUG-3'. Oligonucleotides were labeled using [γ - 32 P]ATP and T4 polynucleotidyl kinase. Specific activities were adjusted to 1–2 $\times 10^3$ c.p.m./fmol DNA. Duplexes were formed by incubation of the appropriate oligonucleotides in 50 mM

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Tris-OAC (pH 7.5), 200 mM NaCl and 20 mM MgCl₂ at 75°C for 2 min followed by slow cooling (1.5 h) to room temperature and spin dialysis (35) to remove the salt.

The 307 nt duplex fragment was isolated from a *Msp*I digest of pBR322 DNA after electrophoresis through a 6% polyacrylamide gel. The appropriate band was excised, the DNA eluted by the crush and soak technique (36) and the DNA recovered by ethanol precipitation. This DNA fragment was labeled by end repair using the Klenow fragment and [α -³²P]dCTP before use.

Annealing assay

Unless indicated otherwise, DNA annealing activity was determined in standard reaction mixtures (20 μ l) containing 40 mM Tris-HCl (pH 7.5), 2 mM ATP, 2 mM MgCl₂, 1 mM DTT, 50 μ g/ml bovine serum albumin and duplex substrate (100 fmol). The reaction mixture was boiled for 5 min and cooled quickly on ice for 5 min before use. τ was added as indicated. Incubations were at 37°C for 10 min. Reactions were terminated by the addition of SDS and proteinase K to 0.5% and 250 μ g/ml respectively, followed by incubation at 37°C for 5 min. Samples were then mixed with a 1/5 vol of a gel loading dye containing 50 mM EDTA, 2% Sarkosyl, 12.5% glycerol, 0.1% xylene cyanol and 0.1% bromophenol blue and the reactions analyzed by electrophoresis through an 8% polyacrylamide gel (19:1 acrylamide:bisacrylamide) (14 \times 14 cm) at 11 V/cm for 1 h using 45 mM Tris, 45 mM boric acid, 1 mM EDTA as the electrophoresis buffer. The gels were dried onto DE-81 paper (Whatman) and autoradiographed. The extent of annealing was quantitated using a Fuji BAS 1000 phosphorimager.

Gel filtration of τ

τ (750 μ g at 3.7 mg/ml) was injected onto a Pharmacia Superose 12 FPLC column that had been equilibrated with 50 mM Tris-HCl (pH 7.5 at 4°C), 2.5 mM DTT, 0.1 mM EDTA, 150 mM NaCl and 20% glycerol. The column was developed in the same buffer at a flow rate of 0.1 ml/min and 0.3 ml fractions were collected.

τ replication assay

Reaction mixtures (25 μ l) containing 25 mM HEPES-KOH (pH 8.0), 50 mM potassium glutamate, 10 mM MgOAc, 1 mM DTT, 20 μ g/ml rifampicin, 2 mM ATP, 200 μ M each of CTP, UTP and GTP, 50 μ M dNTPs containing [³H]TTP (112 c.p.m./pmol), M13Gori1 DNA (457 pmol as nt), 21 pmol SSB, 3 pmol DnaG, 370 fmol core, 520 fmol β , 390 fmol δ , 330 fmol δ' , 330 fmol $\chi\psi$ and the indicated amounts of τ were incubated for 10 min at 30°C. The samples were then precipitated with 5% trichloroacetic acid and the radioactivity retained on glass fiber filters was determined by liquid scintillation spectrometry.

RESULTS

A DNA annealing activity associated with τ

During the course of our continuing analysis of the role of the proteins at the replication fork we noted a DNA annealing activity associated with the τ subunit of the DNA pol III HE. We developed a convenient assay to score this activity, in which duplex formation between two complementary 78mers was assessed by electrophoresis through neutral polyacrylamide gels.

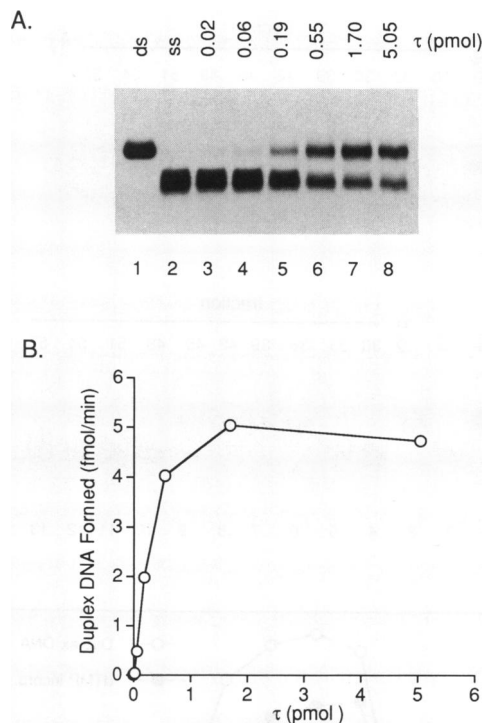


Figure 1. A τ -associated DNA annealing activity. The indicated amounts of τ were added to standard reactions mixtures and incubation, processing and analysis of the reaction were as described under Materials and Methods. (A) Polyacrylamide gel electrophoretic analysis of the reaction products. (B) The extent of duplex DNA formation was quantitated using a Fuji BAS 1000 phosphorimager.

Formation of a 78mer duplex DNA from its constituent single strands was proportional to the amount of τ added to the reaction mixture (Fig. 1). Maximum rates of reaction were observed at molar ratios of τ :total single strands of 5–10:1.

To confirm that this annealing activity was associated with the τ polypeptide, we subjected τ to FPLC gel filtration through a Superose 12 column. Fractions were assayed for the presence of the τ polypeptide, DNA annealing activity and τ DNA replication activity (measured as HE replication activity in the absence of γ on a singly primed phage DNA template) (Fig. 2). DNA annealing activity was found to elute coincident with the τ polypeptide and τ DNA replication activity, thus suggesting that the annealing activity was intrinsic to τ . In addition, the annealing activity present in the column fractions was ablated when they were boiled for 2 min prior to assay (data not shown). Thus the observed annealing activity was not a result of a salt-, metal ion- or polymer-induced increase in the base rate of annealing.

Characterization of the τ DNA annealing activity

τ DNA annealing activity was stimulated 6-fold by 2 mM MgCl₂ (Fig. 3). Higher concentrations inhibited the reaction and increased the rate of τ -independent DNA annealing.

At just a 2-fold molar excess over single strands, τ increased the initial rate of annealing of the complementary single strands by 12-fold (Fig. 4). Annealing of 50% of the substrate was achieved in 20 min under these conditions (Fig. 4).

τ is a single-stranded DNA-dependent ATPase (16), we therefore investigated the effect of ATP and its non-hydrolyzable analogs on the annealing activity (Fig. 5). ATP stimulated the

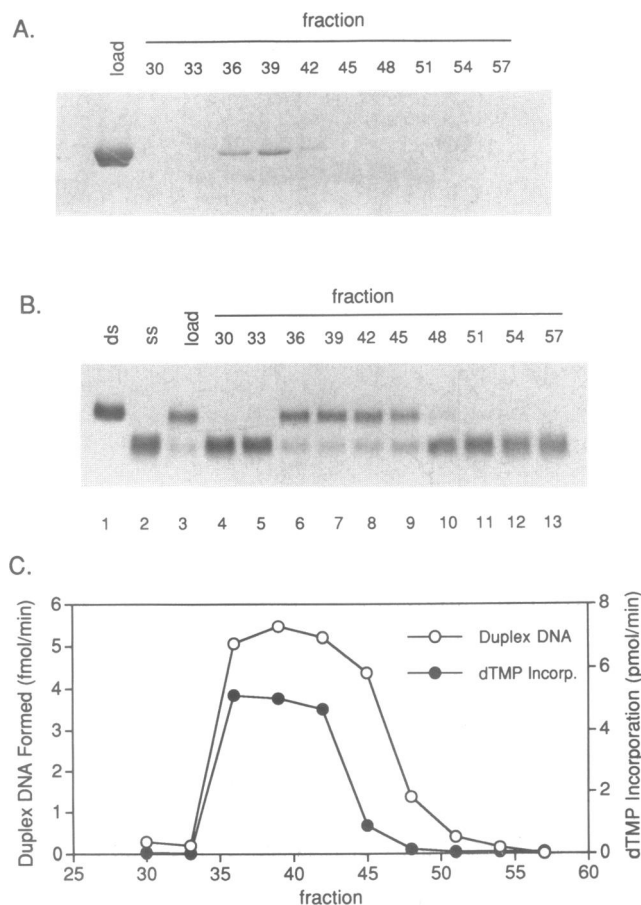


Figure 2. The annealing activity co-elutes during gel filtration with the τ polypeptide and τ DNA replication activity. τ was gel filtered as described under Materials and Methods. The indicated fractions were analyzed by SDS-PAGE for the presence of the τ polypeptide (A) and assayed for either DNA annealing activity (B) or τ DNA replication activity (C) as described under Materials and Methods. Quantitation of the annealing activity is included in (C). The annealing reaction shown in lane 3 of (B) contained 4.5 pmol τ . Fractions 36, 39, 42 and 45 had protein concentrations of 131, 162, 53 and 16 $\mu\text{g/ml}$ respectively. An aliquot of 1/6 μl of each fraction was assayed for activity.

reaction slightly. On the other hand, neither ATP γ S nor AMPPNP inhibited the reaction. This suggested that the mechanism of annealing was not related to the cycle of conformational changes required to elicit ATP hydrolysis, but was more likely a proximity effect arising from the fact that because τ is a dimer (22), complementary oligonucleotides may be brought close together. If τ -mediated DNA annealing arises from this type of proximity effect, then more τ should be required to increase the rate of annealing of longer DNA strands. This proved to be the case.

τ could catalyze the annealing of two 307 nt single-strands (Fig. 6), although the maximum rate of annealing was almost 125-fold less than that observed for the 78mer duplex. A ratio of τ :single strands of almost 200:1 was required to achieve annealing of 50% of the sample in 30 min. Thus it was clear that the efficiency of the τ -mediated annealing fell off rapidly as the size of the DNA increased.

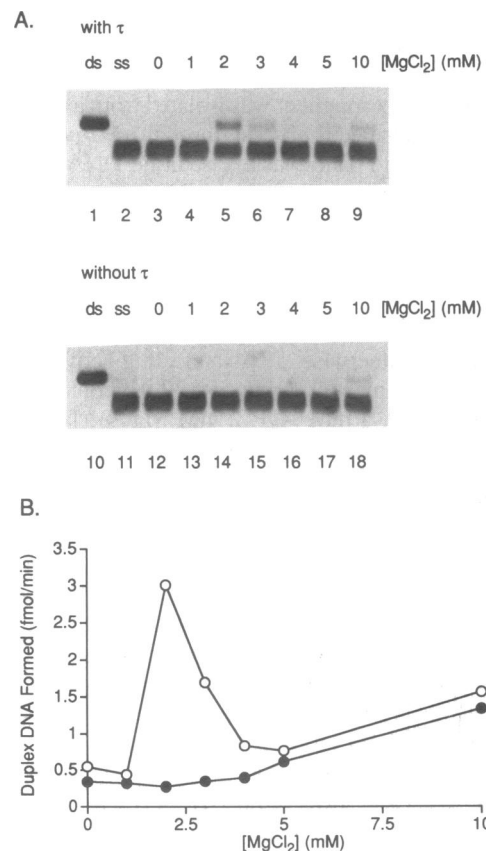


Figure 3. τ DNA annealing activity is stimulated by Mg^{2+} . Standard reaction mixtures containing either no τ (lanes 3 and 12–18) or 200 fmol τ and the indicated concentrations of MgCl_2 were incubated, processed and analyzed as described under Materials and Methods. (A) Polyacrylamide gel analysis of the reaction. Lanes 1 and 10, double-stranded marker; lanes 2 and 11, heat denatured duplex. (B) Quantitation of the extent of duplex DNA formation as described in the legend to Figure 1. \circ , with τ ; \bullet , without τ .

τ can mediate the annealing of an RNA–DNA hybrid

If the DNA annealing activity of τ has a function at the replication fork, the most likely candidate for its action would be the primers on the lagging strand template. We therefore asked whether τ could mediate annealing of a RNA–DNA hybrid. Two complementary 30mers, one RNA, the other DNA, were used for these experiments.

τ successfully mediated annealing of the RNA and DNA single strands (Fig. 7). τ -Independent annealing was higher with these oligonucleotides because they were relatively short, however, it was clear that τ stimulated the rate of annealing. The maximum rate of annealing was roughly half that observed with the DNA 78mers.

DISCUSSION

DNA pol III HE is the multisubunit replicase of *E. coli* (1). The function of many of these subunits at the replication fork can be inferred from studies of the independent activities of the subunits in partial reactions. The core ($\alpha\epsilon\theta$) contains both the catalytic polymerase subunit α (3) and the 3'→5' proofreading

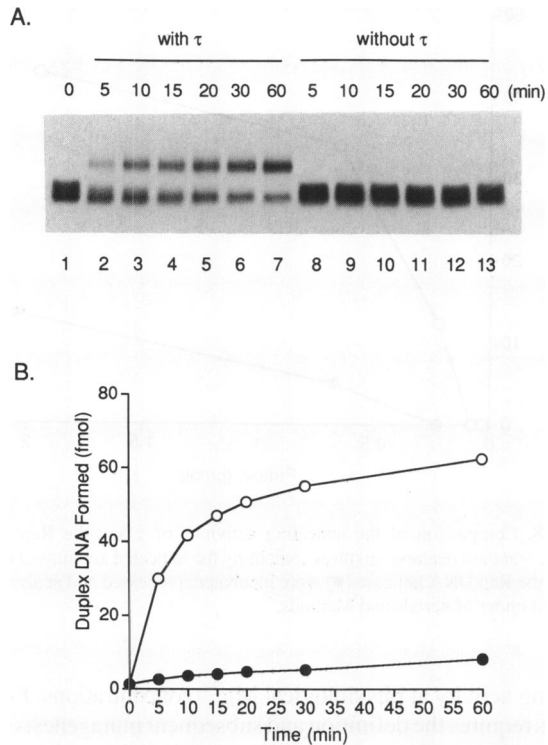


Figure 4. Time course of τ -mediated DNA annealing. Standard reaction mixtures containing either a τ :total single strand ratio of 1:1 (lanes 2–7) or no τ (lanes 8–13) were increased in size 7-fold and incubated at 37°C. Aliquots (20 μ l) were removed at the indicated times, processed and analyzed as described under Materials and Methods. (A) Polyacrylamide gel analysis of the reaction products. Lane 1, heat denatured starting material. (B) Quantitation of the extent of duplex DNA formation. \circ , with τ ; \bullet , without τ .

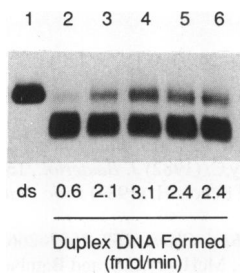


Figure 5. ATP is not required for τ -mediated DNA annealing. Standard reaction mixtures containing either no τ (lane 2) or 200 fmol τ (lanes 3–6) and either no ATP (lane 3) or 2 mM ATP, ATP γ S or AMPPNP (lanes 4–6 respectively) were incubated, processed and analyzed as described under Materials and Methods. Lane 1, double-stranded marker. The extent of duplex formation was quantitated as described in the legend to Figure 1.

exonuclease ϵ (4). The function of θ is not known, although it does interact specifically with ϵ (31).

The core polymerase is a heterotrimer that is made to dimerize in the presence of τ , itself a dimer (22), to form pol III', which has a subunit composition $(\alpha\epsilon\theta)_2\tau_2$ (2). τ is also a single-stranded DNA-dependent ATPase (16) and can substitute for γ , which is produced by a translational frame-shift from the same gene as τ (18–20), in loading the β dimer sliding clamp onto the primer–template (21). γ , which is equivalent to the N-terminal 2/3 of τ (18–20), cannot substitute for τ in dimerizing the core (22).

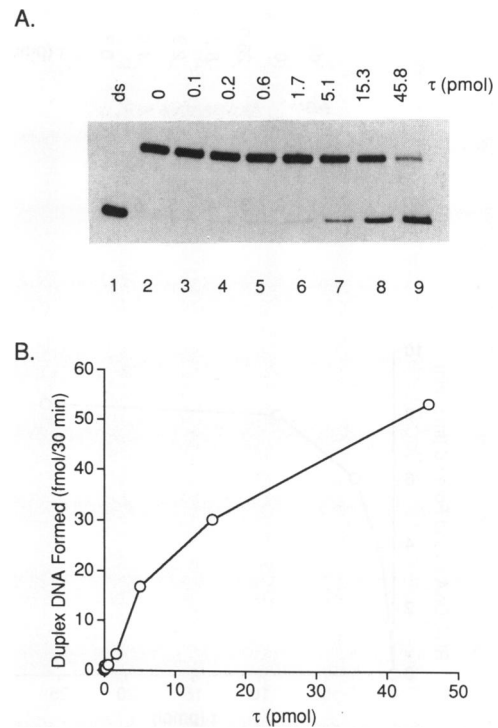


Figure 6. τ -Mediated annealing of 307 nt complementary single strands. Standard reaction mixtures containing 100 fmol of each complementary strand of the 307 bp fragment and either no τ (lane 2) or the indicated amounts of τ (lanes 3–9) were incubated for 30 min at 37°C. Subsequent processing and analysis of the samples was as described under Materials and Methods. (A) Polyacrylamide gel analysis of the reaction products. Lane 1, double-stranded marker. (B) The extent of duplex DNA formed was quantitated as described in the legend to Figure 1.

Loading of the β clamp is catalyzed in an ATP-dependent fashion by the γ complex (21), composed of $\gamma_2\delta\delta'\chi\psi$ (37), although $\gamma_2\delta\delta'$ is nearly as efficient in this reaction as the intact γ complex (38). It has been suggested that $\chi\psi$ acts to stabilize the γ complex (39).

At the replication fork, the active leading and lagging strand polymerases operate functionally as a dimer (23), although it is not clear whether τ is required for this (23). During rolling circle-type DNA replication reconstituted with purified HE subassemblies and the ϕ X-type primosomal proteins, τ was shown to influence the cycling of the lagging strand polymerase from the just-completed Okazaki fragment to the new primer for the next Okazaki fragment (23). We have demonstrated here that τ possesses a DNA annealing activity.

The τ DNA annealing activity was stimulated by Mg^{2+} and did not require ATP. τ stimulated the rate of annealing at ratios to total single strands of 1:1. Significant annealing could be observed even at lower ratios. Thus it seems unlikely that τ was stimulating reannealing in the same manner that some single-stranded DNA binding proteins do, such as SSB (40) and Rep (41), i.e. by coating the phosphodiester backbone of the single strands leading to full exposure of the bases to solution. In support of this, when present at equimolar concentrations the rate of τ -stimulated annealing was 4- to 8-fold greater than the rate of Rep-stimulated annealing (Fig. 8). Thus it is possible that the τ annealing activity arises from the fact that the protein is a dimer. Binding of single strands to each monomer in the τ dimer could raise the local

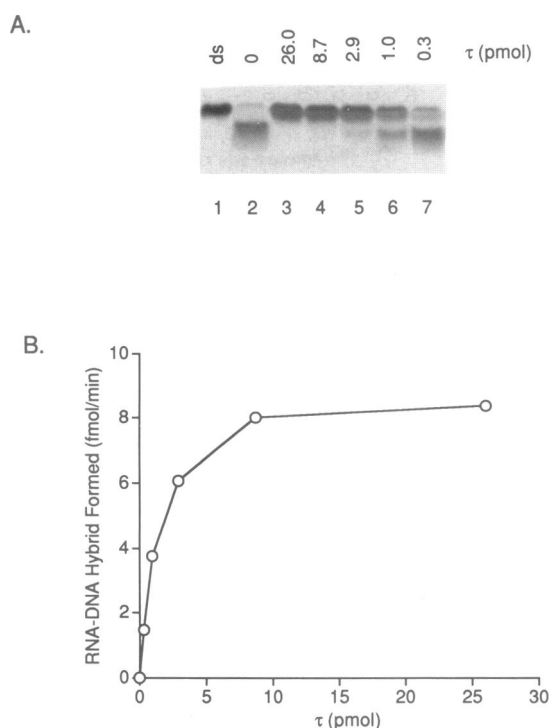


Figure 7. τ -Mediated formation of an RNA-DNA hybrid. Standard reaction mixtures containing 100 fmol each of the 30mer DNA (^{32}P -labeled) and RNA substrates and either no τ (lane 2) or the indicated amounts of τ (lanes 3-7) were incubated, processed and analyzed as described under Materials and Methods. (A) Polyacrylamide gel (10%) analysis of the reaction products. (B) The extent of RNA-DNA hybrid formed was quantitated as described in the legend to Figure 1.

concentration of complementary sequences, thereby increasing the rate of nucleation. The lack of modulation of the annealing activity by ATP would support such a passive role for τ . However, this does not appear consistent with the observation that a 4-fold increase in the size of the DNA substrate resulted in a 100-fold decrease in the rate of annealing. On the other hand, the observed decrease in the rate of annealing with longer substrates could be the result of a kinetic block arising from the existence of hairpin structures in the single-stranded DNA. It is therefore difficult, without additional data, to propose a satisfactory mechanism for τ -stimulated annealing.

How might this annealing activity contribute to the role τ plays at the replication fork? We showed that τ was also able to stimulate annealing of complementary RNA and DNA strands. In principle, therefore, this activity could contribute to stabilization of the RNA primers that are used to initiate Okazaki fragment synthesis on the lagging strand template. These primers, which are on average 10-12 nt long, are known to be relatively unstable at the replication fork (42,43). In addition, the exchange of a primer from DnaG to the lagging strand polymerase is a complicated process that occurs on a platform apparently too short to support the footprint of an initiation complex (44), thus some mechanism may be required to maintain the stability of the primer on the template. It is conceivable that the primer is never actually exposed to solution, but just transferred in a coordinated fashion from protein to protein within the macromolecular replisome. This could explain the apparent lack of τ -stimulated

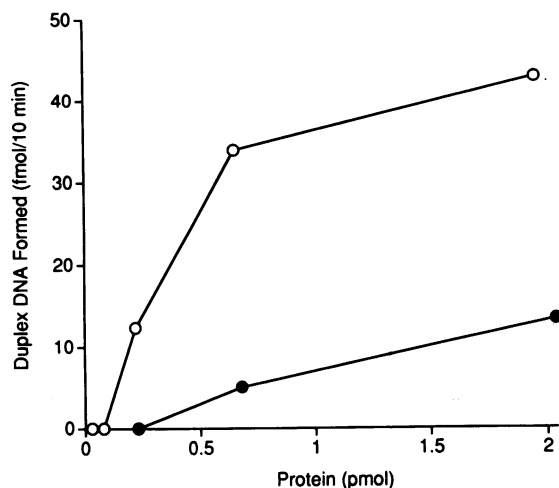


Figure 8. Comparison of the annealing activities of τ and the Rep DNA helicase. Standard reaction mixtures containing the indicated amounts of either τ (○) or the Rep DNA helicase (●) were incubated, processed and analyzed as described under Materials and Methods.

annealing activity at physiological Mg^{2+} concentrations. Further analysis requires the definition and subsequent mutagenesis of the DNA binding site in τ .

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