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The *Escherichia coli* DNA Polymerase III Holoenzyme Contains Both Products of the *dnaX* Gene, τ and γ, but Only τ Is Essential

ALEXANDRA BLINKOVA,[1] CHRISTINE HERVAS,[1] P. TODD STUKENBERG,[2] RENE ONRUST,[2] MICHAEL E. O'DONNELL,[2,3] AND JAMES R. WALKER[1]*

Microbiology Department, University of Texas, Austin, Texas 78712,[1] and Microbiology Department, Cornell University Medical College,[2] and Howard Hughes Medical Institute,[3] New York, New York 10021

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The replicative polymerase of *Escherichia coli*, DNA polymerase III, consists of a three-subunit core polymerase plus seven accessory subunits. Of these seven, τ and γ are products of one replication gene, *dnaX*. The shorter γ is created from within the τ reading frame by a programmed ribosomal −1 frameshift over codons 428 and 429 followed by a stop codon in the new frame. Two temperature-sensitive mutations are available in *dnaX*. The *2016*(Ts) mutation altered both τ and γ by changing codon 118 from glycine to aspartate; the *36*(Ts) mutation affected the activity only of τ because it altered codon 601 (from glutamate to lysine). Evidence which indicates that, of these two proteins, only the longer τ is essential includes the following. (i) The *36*(Ts) mutation is a temperature-sensitive lethal allele, and overproduction of wild-type γ cannot restore its growth. (ii) An allele which produced τ only could be substituted for the wild-type chromosomal gene, but a γ-only allele could not substitute for the wild-type *dnaX* in the haploid state. Thus, the shorter subunit γ is not essential, suggesting that τ can substitute for the usual function(s) of γ. Consistent with these results, we found that a functional polymerase was assembled from nine pure subunits in the absence of the γ subunit. However, the possibility that, in cells growing without γ, proteolysis of τ to form a γ-like product in amounts below the Western blot (immunoblot) sensitivity level cannot be excluded.

Escherichia coli DNA polymerase III (Pol III) holoenzyme consists of a three-subunit core polymerase (α, ε, θ) plus seven accessory subunits (β, τ, γ, δ, δ′, χ, ψ) (20, 31, 33, 54). Study of subassemblies and pure subunits has advanced the understanding of accessory subunit function. In an ATP-dependent reaction, the γ complex (γ, δ, δ′, χ, ψ) catalyzes the transfer of β to the primed template to form a preinitiation complex (10, 27, 37, 39, 52). This preinitiation complex consists of a β dimer which completely encircles the DNA and slides freely along the duplex (19, 47). The core or individual α (DNA polymerase) (25, 44) and ε (proofreading exonuclease) (42) subunits then bind and polymerize with high processivity, tethered to the template by the β dimer clamped around the duplex DNA behind α (11, 19, 47).

Both τ and γ are produced from one gene, *dnaX* (8, 13, 18, 35, 62). The 71.1-kDa τ is the full-length 643-amino-acid translational product of the *dnaX* messenger. The 47.5-kDa γ is terminated within the reading frame by a programmed ribosomal −1 frameshift over codons 428 and 429 (2, 9, 48, 49, 51). The shifted ribosomes incorporate one unique amino acid and then encounter a stop codon. The result is that γ is identical to the first 430 residues of τ plus a unique C-terminal residue. The frameshift signal is so efficient that the τ/γ ratio in nonoverproducing strains is about 1 (2, 9, 22, 51).

Although not required for processive synthesis in reconstituted systems which included the other nine subunits, addition of τ stimulated total synthesis (27, 28). Moreover, heterodimers of γδ, τδ, or τδ′ (but not γδ′) can substitute for the entire γ complex in catalyzing preinitiation complex formation (36). Hence, in the absence of γ, τ can substitute for the γ function in placing β on DNA. A major feature of τ which γ does not have is the ability to bind directly and, thereby, dimerize the α polymerase (32, 45). Therefore, the C-terminal residues of τ which are lacking in γ are essential for the polymerase interaction.

In this paper, we address the roles played by the structurally related τ and γ proteins. By several criteria, τ is shown to be essential, but γ is not, under normal growth conditions. This suggests that τ can substitute for the normal function(s) of γ.

MATERIALS AND METHODS

Media. Yeast extract-tryptone medium (15) was supplemented with 0.5% NaCl except that, for growth of strains GM36 and RM36, no NaCl was added. Ampicillin, tetracycline, kanamycin, chloramphenicol, or spectinomycin (200, 15, 50, 30, or 50 μg/ml, respectively) was added, as necessary.

Strains and plasmids. *E. coli* K-12 strains and principal plasmids are listed in Table 1. M13mp9, M13mp19, and pUC19 (60) were used. The low-copy-number plasmid pCL1920 (24) was obtained from Masayori Inouye. pMAK705 (12) was from Sidney Kushner. pAB8 was constructed by cloning a 2.8-kb *Hin*dIII-*Pst*I *dnaX*$^+$ region (Fig. 1) of pJH16 (35) into pUC19. pAB18 was constructed by cloning a 2.8-kb *Hin*dIII-*Pst*I *dnaX*$^+$ fragment plus the *Pst*I-*Bam*HI region of M13mp9 polylinker from an M13mp9 hybrid derivative into pBR322. pAB45 was constructed from pBJ1 (61), which is the 6.3-kb *dnaX* region cloned into pBR322, by subcloning the 3.9-kb *Hin*dIII-*Kpn*I *dnaX* region into pMAK705 (12). pAB46 was prepared by deleting the 650-bp *Bst*EII fragment from pAB45. pAB47 and pAB48

* Corresponding author.

TABLE 1. Strains

Bacterial strain	Relevant genotype or phenotype	Source or reference
C600	*dnaX*$^+$	Barbara Bachmann
Hfr8	Transfers *dnaX*$^+$ early	4
CBK0301	*purE*::Tn*5* *dnaX*$^+$	Claire Berg
AX727	*dnaX2016*(Ts)	7
AX727/F'*lac*	Male, *dnaX2016*(Ts)	This study
AX800	*purE*::Tn*5* *dnaX2016*(Ts)	This study
AX801	*recA*::Tn*10* *dnaX2016*(Ts)	This study
GM36	*dnaX36*(Ts)	14
AX810	*recA*::Tn*10* *dnaX36*(Ts)	This study
AX820	*purE*::Tn*5* *dnaX2016*(Ts) derivative of Hfr8	This study
AB500	*dnaX*(GCA428GCG; AAA429AAG)	This study
AB501	*dnaX*$^+$	This study
JM103Y	Host for M13mp19	E. E. Snell

were constructed by recloning the wild-type and deleted *dnaX* fragments from pAB45 and pAB46, respectively, into pUC19. pAB41 contains a mutant *dnaX* allele altered to eliminate the ribosomal frameshift signal by changing the GCAAAA sequence over codons 428 and 429 to GCGAAG. This change is designated GCA428GCG; AAA429AAG. This plasmid was constructed by recloning the GCA428GCG; AAA429AAG mutant *Hin*dIII-*Pst*I *dnaX* region of mutated M13 derivative AB5 (2) into pMAK705. Construction of other plasmids is described below and in Results. AB15 is an M13mp19 derivative which contains a cloned 2.8-kb *Hin*dIII-*Pst*I *dnaX36*(Ts) fragment from strain GM36 (14); single-strand DNA from this phage is identical to *dnaX* messenger.

Amplification by the polymerase chain reaction. The *36*(Ts) allele was amplified from genomic DNA (57) of strain GM36 (14) with the GeneAmp Kit (Perkin-Elmer Cetus, Norwalk, Conn.). The primers (5' 301 to 317 and 5' 3083 to 3067) were complementary to the *Hin*dIII and *Pst*I regions and produced a 2.8-kb fragment which was cloned into *Hin*dIII- and *Pst*I-cut M13mp19. Single-strand DNA of this phage, designated AB15, was used for sequencing. The 2.8-kb *Hin*dIII-*Pst*I *dnaX36*(Ts) fragment cloned into pBR322 was designated pAB33; cloned into the low-copy-number derivative of pSC101, pCL1920 (24), it was designated pAB33a.

Marker rescue. Rescue of the *2016*(Ts) mutation was tested by infecting *dnaX2016*(Ts) strain AX727/F' *lac* at 30°C with M13 derivatives containing *dnaX* fragments, incubating them at 30°C for 1 h, and plating the infected cells at 42°C. Rescue of the *36*(Ts) mutation was tested by transforming *dnaX36*(Ts) strain GM36 with pBR322-containing *dnaX* fragments at 30°C, growing transformants at 30°C, and determining plating efficiency at 43°C.

Extracts. Whole-cell extracts were prepared for Western blots (immunoblots) by boiling in sodium dodecyl sulfate (SDS). Ten-milliliter cultures were incubated for 16 h, centrifuged, resuspended in 10 ml of Tris (10 mM [pH 7.5]), centrifuged, resuspended in 250 μl of Tris (10 mM [pH 7.5])–EDTA (1 mM)–SDS (1%), and boiled for 2 min.

Western blot analysis. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Schleicher & Schuell nitrocellulose membrane (PH79; 0.1-μm pore size) overnight with the Bio-Rad Trans Blot apparatus, and detected with polyclonal antibody to γ (prepared as described by Stukenberg et al. [47]) which reacts with both τ and γ (5). The nitrocellulose was blocked with 10% nonfat dry milk for 1 to 1.5 h, was exposed to specific antibody, and then was exposed to phosphatase-labelled goat anti-rabbit immunoglobulin G (Kirkegaard Laboratories).

Purification of Pol III* without γ. Three hundred liters of AB500 cells was grown to an optical density of 4 at 37°C in Luria broth supplemented with glucose, thymine, and thiamine (55). Cells were cooled, harvested by centrifugation (3.7 kg), lysed with lysozyme (fraction I), and fractionated by ammonium sulfate (fraction II) as described previously (55). The procedures which follow were performed at 4°C unless otherwise specified. Fraction II (466 mg of protein) was resuspended in a total volume of 30 ml of buffer A (20% glycerol, 20 mM Tris, 0.2 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), dialyzed to a conductivity equal to 250 mM NaCl, diluted with 70 ml of 20% glycerol to a conductivity equal to 55 mM NaCl, clarified by centrifugation, and fractionated by heparin agarose column chromatography as described previously (29). The heparin agarose fractions with greater than half the peak activity were pooled (fractions 20 to 29; 90 ml, 57 mg) to give fraction III. Fraction III was dialyzed against buffer A to a conductivity of 39 mM NaCl, fractionated on an 8-ml MonoQ column (Pharmacia-LKB), and developed with a 196-ml linear gradient of 0 to 500 mM NaCl in buffer A. Eighty fractions were collected and assayed, and fractions with greater than half the peak of Pol III* activity were pooled (fractions 50 to 54; 15 ml, 2 mg) to give fraction IV. Fraction IV was dialyzed against buffer A to a conductivity equal to 20 mM NaCl, loaded onto a 2-ml ATP-agarose column (linked through the N-6 position of adenosine [Sigma]), washed with 15 ml of buffer A plus 125 mM NaCl, and eluted with buffer A plus 2 M NaCl to yield fraction V (3 ml, 0.36 mg). Only 6% of Pol III* activity was recovered during the ATP-agarose column step. The core polymerase activity flowed through the ATP-agarose column, and the eluted material contained activity equivalent to that of the γ complex. Analysis of fraction V on a Coomassie blue-stained 12% polyacrylamide gel showed the presence of δ, δ', χ, and ψ subunits as well as the presence of a γ-like protein. Activity assays were performed as follows. Pol III* activity was measured by the ability to combine with β in extension of a single DNA 30-mer completely around an M13mp18 single-stranded circular DNA coated with single-strand-binding protein as described by Onrust et al. (39), except that preincubation of protein with DNA was for 2 min instead of 8 min, prior to initiating a 20-s pulse of synthesis by adding dATP and [α-^{32}P]dTTP. Pol III core activity was measured as described for Pol III*, except that 5 ng of γ complex was added to the preincubation mixture. γ complex activity was assayed as described for Pol III* activity, except that 44 ng of αε complex was added to the preincubation mixture.

Gel filtration. Gel filtration of the αεθτδδ'χψ complex was performed with an HR 10/30 Superose 6 column (Pharmacia-LKB) equilibrated in buffer B (50 mM Tris-HCl [pH 7.5], 10% glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, and 0.2 mM ATP).

Constitution of Pol III* lacking γ. The τ subunit (239 μg, 0.84 nmol as dimer), χ (40.5 μg, 2.44 nmol as monomer), and ψ (30 μg, 1.97 nmol as monomer) were incubated for 30 min at 15°C, at which time δ (92 μg, 2.38 nmol as monomer) and δ' (73.7 μg, 1.99 nmol as monomer) were added. This mixture was further incubated for 30 min at 15°C, at which time α (239 μg, 1.85 nmol as monomer), ε (67.6 μg, 2.46 nmol as monomer), and θ (24.13 μg, 2.8 nmol as monomer) were added to a final volume of 260 μl containing 5 mM $MgCl_2$ and

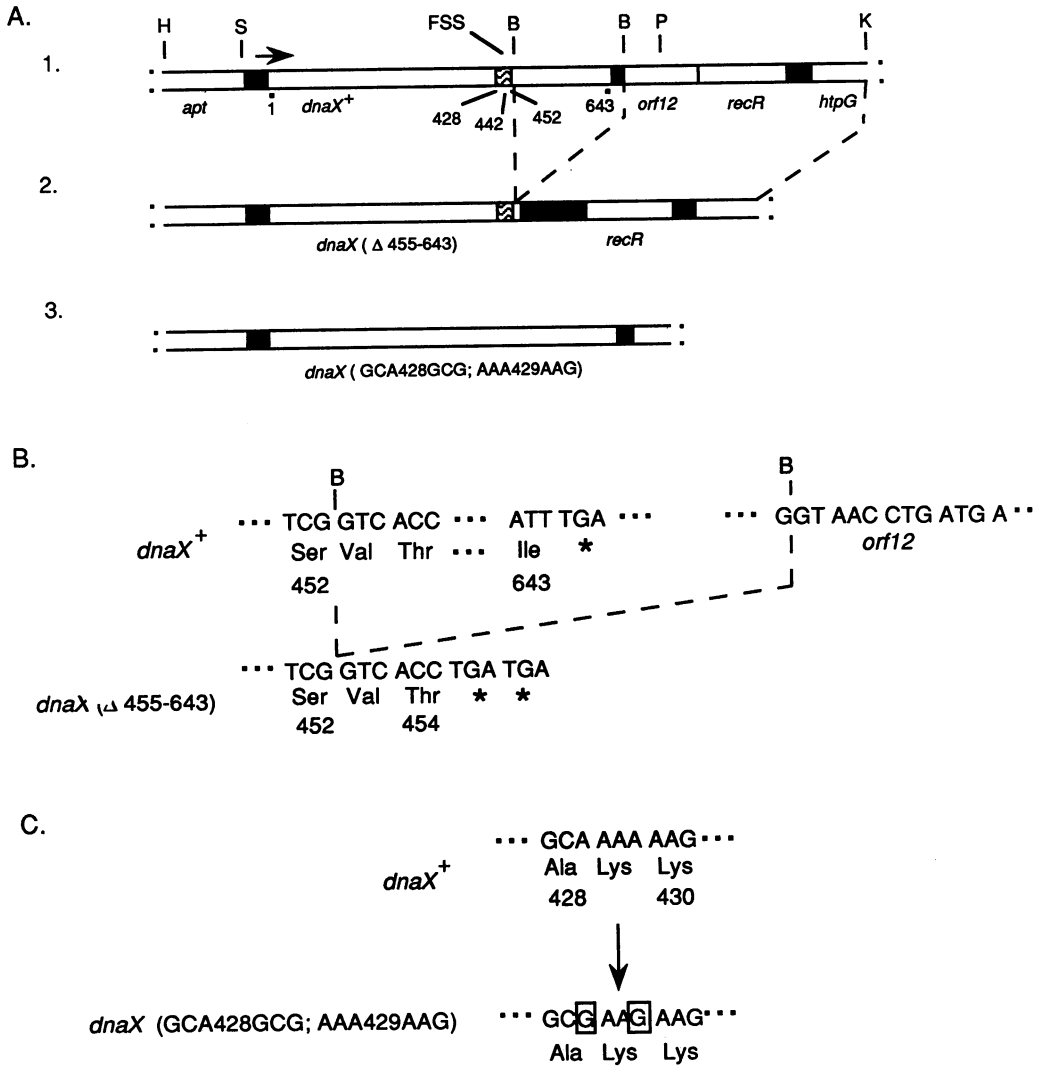

FIG. 1. Construction of *dnaX* alleles which produce only γ or only τ. (A) Maps. Open bars refer to reading frames; darkened areas are intergenic regions; the hatching marks a programmed ribosomal frameshift signal (FSS); a vertical bar marks overlapping reading frames. Map 1 shows the 3.9-kb *Hin*dIII-*Kpn*I fragment carrying the 3′ end of *apt*, the *dnaX*, *orf12* and *recR* genes, and the 5′ portion of *htpG* (61). The numbers refer to *dnaX* codons. Map 2 shows a γ-only allele *dnaX*(*Δ455-643*) formed by deleting the *Bst*EII fragment which carries the *dnaX* 3′ end from pAB18, forming pAB31. The sequence of the rejoined fragment was confirmed by sequencing. Map 3 shows a 2.8-kb *Hin*dIII-*Pst*I τ-only fragment on which the frameshift signal was eliminated without altering the amino acid codes [*dnaX*(GCA428GCG; AAA429AAG)] and which was cloned from an M13 derivative AB5 (2) into pBR322, forming pAB34, into pCL1920, forming pAB34a, or into pMAK705, forming pAB41. (B) Sequences of portions of wild-type *dnaX* and *orf12* and the γ-only allele *Δ455-643*. Cut sites for *Bst*EII are indicated by B and the vertical bars. (C) Sequences of *dnaX*$^+$ and the τ-only mutant allele (GCA428GCG; AAA429AAG) over codons 428 to 430 (2). The mutations are boxed. H, *Hin*dIII; S, *Sma*I; B, *Bst*EII; P, *Pst*I; K, *Kpn*I.

0.2 mM ATP. This mixture was further incubated for 30 min at 15°C and was then concentrated to 100 μl at 4°C with a Centricon 30 (Amicon). The entire sample was injected onto the Superose 6 column. Proteins were purified as described in the following references: α, ε, and τ, 39; δ and δ′, 38; χ and ψ, 59; θ, 46. The column was developed with buffer B at 0.3 ml/min and after the first 6.85 ml, 200-μl fractions were collected. Fractions were analyzed by SDS-polyacrylamide gels (15% polyacrylamide [100 μl per lane]) and stained with Coomassie blue. Replication assays of column fractions were performed as described previously (39), except that the replication mix contained only the β subunit and DNA synthesis was for 4 min. An aliquot of column fraction (2 μl) was added to the assay after a 20-fold dilution with 20 mM Tris-HCl (pH 7.5)–10% glycerol–2 mM dithiothreitol–0.5 mM EDTA–50 μg of bovine serum albumin per ml. Protein standards (Bio-Rad and Sigma) were a mixture of 50 μg each in 10 μl of buffer B.

Other techniques. The method of Willetts et al. (56) was used for transduction by P1 *vira*. Exponentially growing donors and exponentially growing or stationary-phase recipients were mixed with gentle shaking for conjugation. The method of Lederberg and Cohen (21) was used for transformation. Standard recombinant DNA techniques (30) were used. Single-strand or plasmid (6) DNA was sequenced (41) with the Sequenase kit from U.S. Biochemicals.

TABLE 2. Plasmids

Plasmid	Insert	*dnaX*	Vector
pAB8	2.8-kb *Hin*dIII-*Pst*I	Wild type	pUC19
pAB18	2.8-kb *Hin*dIII-*Pst*I[a]	Wild type	pBR322
pAB19	2.8-kb *Hin*dIII-*Pst*I[a]	*2016*(Ts)	pBR322
pAB20	2.8-kb *Hin*dIII-*Pst*I[a]	Wild-type temperature-sensitive revertant of *2016*(Ts)	pBR322
pAB31	2.1-kb, 650-bp *Bst*EII-*Bst*EII deletion of pAB18[a]	*Δ455-643*	pBR322
pAB33	2.8-kb *Hin*dIII-*Pst*I	*36*(Ts)	pBR322
pAB34	2.8-kb *Hin*dIII-*Pst*I[b]	GCA428GCG; AAA429AAG	pBR322
pAB33a	2.8-kb *Hin*dIII-*Pst*I	*36*(Ts)	pCL1920
pAB34a	2.8-kb *Hin*dIII-*Pst*I	GCA428GCG; AAA429AAG	pCL1920
pAB41	2.8-kb *Hin*dIII-*Pst*I	GCA428GCG; AAA429AAG	pMAK705
pAB45	3.9-kb *Hin*dIII-*Kpn*I	Wild type	pMAK705
pAB46	3.3-kb, 650-bp *Bst*EII-*Bst*EII deletion of pAB45	*Δ455-643*	pMAK705
pAB47	3.9-kb *Hin*dIII-*Kpn*I	Wild type	pUC19
pAB48	3.3-kb, 650-bp *Bst*EII-*Bst*EII deletion from pAB45	*Δ455-643*	pUC19

[a] Also contains a *Pst*I-*Bam*HI portion of M13mp9 polylinker.
[b] Also contains a *Pst*I-*Bam*HI portion of M13mp19 polylinker.

RESULTS

Cloning the *dnaX2016*(Ts) and *36*(Ts) alleles. The *dnaX2016* (Ts) allele was cloned from strain AX727 (7) by the procedure of Park and Hazelbauer (40). First, a closely linked, selectable marker, Tn*5*, which carries Kan^r, was moved from the *purE*::Tn*5* *dnaX*$^+$ strain CBK0301 by P1 transduction into the *purE*$^+$ *dnaX2016*(Ts) strain AX727. P1 transduction then moved both *purE*::Tn*5* and *dnaX*(Ts) into the Hfr8 strain (which transfers *purE* and *dnaX* early [4]), forming strain AX820. pAB18, which carries a *dnaX*$^+$ *Hin*dIII-*Pst*I fragment cloned into pBR322 (Table 2), was transformed into the Hfr8 derivative. The nonconjugative pAB18 was transferred by the Hfr into the *dnaX2016*(Ts) strain AX727. Two percent of the transferred plasmids carried the *2016*(Ts) mutation. (This plasmid transfer process is thought to occur as the result of homologous recombination between the hybrid plasmid and the Hfr chromosome. If conducted plasmids undergo two recombination events, one for integration and the second for resolution after transfer, some of the conducted plasmids will have acquired the chromosomal allele [40].) One of the *dnaX2016*(Ts) derivatives of pAB18 was designated pAB19 (Table 2). A revertant *dnaX*$^+$ allele was similarly cloned starting with pAB19 and a revertant strain; the plasmid carrying the revertant allele was designated pAB20 (Table 2).

The *36*(Ts) allele was amplified from genomic DNA of strain GM36 as a 2.8-kb *Hin*dIII-*Pst*I fragment and cloned (Materials and Methods) into pBR322, forming pAB33 or into pCL1920, forming pAB33a.

Sequence of *dnaX2016*(Ts) and *36*(Ts) alleles. The *2016*(Ts) mutation was first located between codons 116 and 147 by marker rescue and then was sequenced. Marker rescue was tested with a series of overlapping fragments described by Yin et al. (62). These fragments were cloned into phage M13mp9, the phages used to infect an episome-containing *dnaX2016*(Ts) mutant, and marker rescue was tested by plating the infected cells at 42°C. Temperature-resistant recombinants formed when the cloned fragments extended to codon 147 but did not form if they terminated before codon 116 (Fig. 2).

The *dnaX2016*(Ts) allele cloned into pBR322, pAB19, was sequenced between nucleotides 1088 and 1303 (on the strand complementary to mRNA) by using the double-strand plasmid as template and a primer complementary to nucleotides 1340 to 1326. One change was observed; codon 118 was changed from GGT (glycine) to GAT (aspartate) and was designated G118D. This change was confirmed as the mutation because, in a revertant allele, pAB20, codon 118 had reverted to the wild type. Thus, the *2016*(Ts) allele produces mutant γ and τ.

The *Ts*(36) allele was located downstream of nucleotide 2377 by marker rescue (Fig. 2). The region from nucleotide 2377 to 2815 was sequenced along one strand by using

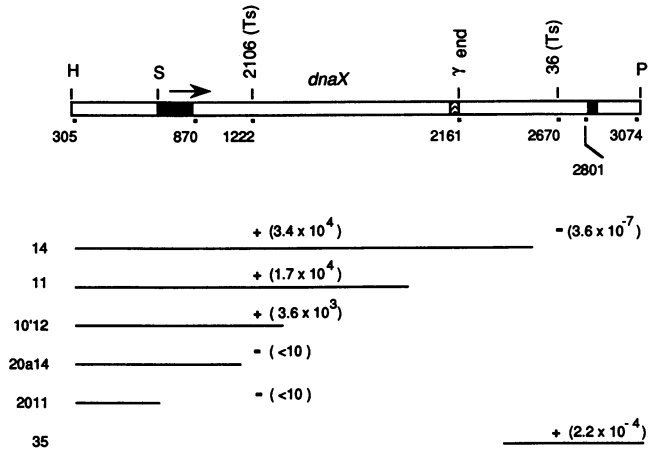

FIG. 2. Marker rescue of *dnaX2016*(Ts) and *36*(Ts) alleles. The 2.8-kb *Hin*dIII-*Pst*I *dnaX* region (open reading frame) is indicated at the top. The arrow indicates direction of *dnaX* transcription. The hatched area is the ribosomal frameshift signal. The positions of the temperature-sensitive mutations and of the γ C-terminal codon are indicated. Nucleotides (below the bar) are numbered from the *Eco*RI site upstream of *dnaX* (8, 62). The lower part describes fragments tested for ability to rescue the temperature-sensitive mutations. For testing rescue of the *2016*(Ts) allele, the fragments extending from the *Hin*dIII site rightward were tested as clones in M13mp19. For testing rescue of the *36*(Ts) allele, fragments 14 and 35 were cloned into pBR322. Fragment numbers are indicated at the left. The results of marker rescue are indicated as + (positive) or − (negative) adjacent to the fragment tested. Qualitative results were confirmed quantitatively. Numbers in parentheses are recombinants per milliliter after infection of the *dnaX2016*(Ts) strain AX727/F′*lac* with M13mp19 derivatives or efficiency of plating at 43°C, relative to 30°C, of *dnaX36*(Ts) cells transformed with pBR322 derivatives.

TABLE 3. Complementation of *dnaX*(Ts) mutants[a]

Plasmid, genotype, and products provided (γ/τ)	Complementation and efficiency of plating at 43°C[b] of *dnaX* recipient:			
	2016(Ts)[c] G118D[d]		*36*(Ts)[e] E601K[f]	
	recA$^+$	*recA*	*recA*$^+$	*recA*
pBR322 derivative				
pBR322 vector (−/−)	− (6×10^{-7})	− (6×10^{-7})	− (5×10^{-7})	− (1×10^{-6})
pAB18 *dnaX*$^+$ (+/+)	+ (1.0)	+ (1.0)	+ (1.0)	+ (0.8)
pAB19 *2016*(Ts) (Ts/Ts)	− (1×10^{-8})	− (6×10^{-6})	− (2×10^{-3})	− (2×10^{-5})
pAB31 Δ*455-643* (+/−)	− (1.7×10^{-4})	− (6×10^{-6})	− (8×10^{-6})	− (8×10^{-5})
pCL1920 derivative				
pCL1920 vector (−/−)	− (6×10^{-7})	NT[g]	− (2.4×10^{-5})	− (6×10^{-7})
pAB33a *36*(Ts) (+/Ts)	NT[h]	NT[h]	− (1.3×10^{-6})	− (5.9×10^{-7})
pAB34a GCA428GCG; AAA429AAG (−/+)	+ (1.0)[i]	NT	+ (1.0)	+ (1.1)

[a] The results of complementation are indicated as + (positive) or − (negative) throughout.
[b] Relative to 30°C.
[c] In strain AX727 and a *recA* derivative.
[d] Provides mutant τ and mutant γ.
[e] In strain GM36 and a *recA* derivative.
[f] Provides mutant τ and wild-type γ.
[g] NT, not tested.
[h] Not tested because the *dnaX36*(Ts) E601K allele is suppressed by addition of NaCl to culture media (14) and the wild-type ancestor of strain AX727 did not plate on media without added NaCl at 43°C.
[i] pAB34a is unstable in this host; complementation was tested on medium containing spectinomycin.

primers (complementary to messenger) covering the sequences 2588 to 2602 5′ and 2847 to 2863 5′. Only one change from the wild type was observed; nucleotide 2670 had changed from G to A, altering the wild-type codon 601 from glutamate to lysine (E601K). This change is downstream of the γ-coding region and influences, directly, the activity of only the τ subunit. This one change was confirmed as the *36*(Ts) mutation because three fragments were cloned after each of two independent amplifications, and this same change was found in all six constructs. Thus, the *36*(Ts) mutant produces mutant τ altered in codon 601, but presumably wild-type γ. The temperature sensitivity of this mutant suggests that τ is essential for normal growth.

Complementation by mutant *dnaX* alleles. The ability of cloned wild-type and mutant alleles to complement specific *dnaX* mutations was tested to gain information about the roles of τ and γ. In addition to the cloned *2016*(Ts) G118D and *36*(Ts) E601K alleles, a deletion which removed the C-terminal 189 codons of the *dnaX* reading frame was constructed. This allele, *dnaX*(Δ*455-643*), was generated by removing the 650-bp *Bst*EII fragment over the 3′ end of *dnaX* in pAB18. This deletion and rejoining reestablished the τ sequence over codons 453 to 454 but then introduced tandem stop codons (Fig. 1). Inasmuch as the *Bst*EII site is downstream of the frameshift signal, this deleted allele produced normal γ and a truncated τ called τ1-454. Another plasmid produced τ, but no detectable γ, as the result of two changes in the programmed ribosomal frameshift signal. The run of six A's over codons 428 to 430 was interrupted by changing codons 428 and 429 from GCA to GCG and from AAA to AAG, respectively (Fig. 1), changes which did not influence the amino acid sequence. These changes have been shown to eliminate detectable γ synthesis (2, 49). This mutant allele (GCA428GCG; AAA429AAG), which produced τ only (Fig. 1), was cloned into pBR322 and was designated pAB34; cloned into pCL1920 it was designated pAB34a.

The wild-type allele complemented both the *2016*(Ts) G118D and *36*(Ts) E601K mutants in both *recA*$^+$ and *recA* backgrounds (Table 3, pAB18). Thus, the presence of mutant proteins did not inhibit the activities of the wild-type factors.

The *2016*(Ts) G118D allele, which alters both τ and γ, complemented neither the *2016*(Ts) nor the *36*(Ts) recipients (Table 3, pAB19). This confirms the requirement for τ because the *36*(Ts) mutant is expected to synthesize γ normally and suggests, furthermore, that overproduction of mutant τ could not complement the resident *36*(Ts) mutation. That γ cannot substitute for τ was also shown by pAB31. This plasmid contains an allele which is deleted for the C-terminal 189 codons of *dnaX*; it directs the synthesis of γ and a truncated τ called τ1-454. This plasmid could not restore growth at high temperatures to the *36*(Ts) mutant which was altered within the τ-specific region at codon 601 (Table 3, pAB31). Moreover, the provision of additional wild-type γ to a strain lacking active τ could not restore growth (Table 3, pAB33a). Plasmid pAB33a carries the *36*(Ts) allele (E601K) and produces active γ; it did not complement the *36*(Ts) recipient, and, in summary, τ seems essential.

Additionally, providing active τ without γ from plasmid pAB34a to a temperature-sensitive strain which produces wild-type γ and temperature-sensitive τ restored growth (Table 3, pAB34a). This τ-only plasmid, pAB34a, fails to direct γ synthesis because the frameshift signal has been eliminated (2). The τ-only pAB34a also complemented the *2016*(Ts) mutant, which has defective γ and τ (Table 3, pAB34a). Thus, cells with τ alone, without active γ, are able to grow. Although the τ-only mutant plasmid complemented the *2016*(Ts) mutant with a plating efficiency of 1.0, this plasmid was unstable in this host, the complementation had to be scored on antibiotic-containing medium, and the colonies at 43°C grew slowly and had small diameters.

Replacement of the chromosomal *dnaX* allele by an allele which produces only γ is not possible (unless *dnaX*$^+$ is provided in *trans*). Gene transplacements were done in the *dnaX*$^+$ strain C600 with the temperature-sensitive suicide vector pMAK705 (12). pMAK705 carries a polylinker region and Cm^r determinant and is replication defective at 44°C. Plasmids pAB45 and pAB46 carry the *dnaX*$^+$ and

TABLE 4. Curing of partial diploid strains

Expt	*dnaX* allele present on:		*dnaX*$^+$ AB18 present[a]	Fraction of Cm^s cells after 10–12 generations at 44°C
	Chromosome	Cm^r suicide vector		
1	Wild type	*Δ455-643*	–	208 of 210[b]
2	*Δ455-643*	Wild type	–	0 of 246[b]
3	Wild type	*Δ455-643*	+	371 of 380[b]
4	*Δ455-643*	Wild type	+	305 of 308[b]
5	Wild type	GCA428GCG; AAA429AAG	–	199 of 200[b,c]
6	GCA428GCG; AAA429AAG	Wild type	–	197 of 200[b,d]

[a] Compatible with the suicide vector. + indicates presence; – indicates absence.
[b] Surviving Cm^r strains no longer contained plasmids; they were cointegrates.
[c] One of the cured strains was designated AB501; it should be identical to strain C600.
[d] One of the cured strains was designated AB500.

dnaX(*Δ455-643*) alleles, respectively, on 3.9- and 3.3-kb *Hin*dIII-*Kpn*I fragments cloned into the *Hin*dIII-*Kpn*I sites of pMAK705.

The plasmid producing only γ, pAB46, was transformed into the *dnaX*$^+$ strain C600 and cointegrates selected by plating directly for Cm^r transformants able to grow at 44°C. About 15 cointegrates (verified by absence of plasmid DNA) were isolated per μg of plasmid DNA. The cointegrates were then allowed to resolve spontaneously during three cycles of growth for 12 generations each in liquid medium plus chloramphenicol at 30°C. Plasmids formed by resolution were identified as *dnaX*$^+$ or *dnaX*(*Δ455-643*) by restriction analysis, the ratio of wild-type to deletion allele being approximately 1:1. If the resolved plasmid was *dnaX*$^+$, the chromosomal allele was inferred to be *dnaX*(*Δ455-643*), an inference which was confirmed later.

Plasmid-containing strains with *dnaX*$^+$ on the chromosome and *dnaX*(*Δ455-643*) on the plasmid (and vice versa) were incubated in liquid medium without chloramphenicol at 44°C to prevent plasmid replication. After 10 to 12 generations at 44°C, the cells were plated at 30°C with and without chloramphenicol to score the fraction cured. When *dnaX*(*Δ455-643*) was carried on the plasmid and the wild type was carried on the chromosome, the plasmid readily was cured. About 99% of the clones present after the 44°C incubation had lost the plasmid and had become Cm^s (Table 4, expt 1). When, however, the chromosomal allele was *dnaX*(*Δ455-643*) and the plasmid carried *dnaX*$^+$, curing was not possible (Table 4, expt 2). The clones which survived the 44°C incubation and remained Cm^r no longer contained plasmids in the cytoplasm; they were cointegrates.

dnaX$^+$ plasmids in the strain carrying *dnaX*(*Δ455-643*) on the chromosome could readily be cured, however, when a wild-type *dnaX* gene copy was provided on a second, compatible, nonthermosensitive plasmid, pAB18 (Table 4, expt 3 and 4). These data suggest that τ, and specifically one or more of the domains present in residues 455 to 643, is essential.

Verification that the γ-only allele, *Δ455-643*, directed synthesis of γ but not τ was sought by Western blot analysis. To be able to measure τ or γ production from the plasmid-borne *dnaX* alleles without interference from the chromosomal copy of the gene, wild-type and γ-only alleles were cloned into the high-copy-number vector pUC19 (60), generating pAB47 and pAB48, respectively. Extracts of strain C600 containing these plasmids were electrophoresed, transferred to nitrocellulose, and probed with polyclonal antiserum which reacts with both τ and γ. From this vector, τ and γ were produced from the wild-type gene on pAB47. Although most of the protein from the plasmid was γ (49), the overproduced τ was consistently observed as a more intense band than that of the extracts of strain C600 or C600 containing pUC19 (Fig. 3, lanes 1 and 4). The strain carrying the γ-only allele in pAB48 produced a very large amount of γ, a small amount of the 49.9-kDa truncated τ1-454, and a very small amount of τ (Fig. 3). The wild-type τ is presumed to be of chromosomal gene origin because its amount was similar to that in the same amount of extract from strain C600.

The chromosomal *dnaX* allele can be replaced by an allele which produces only τ. A pMAK705 derivative which carries the τ-only allele *dnaX*(GCA428GCG; AAA429AAG), pAB41 (Table 2 and Fig. 1), was used for replacement of the resident wild-type allele in strain C600 by the procedure described above. In this experiment, however, the chromosomal, wild-type allele was readily replaced by the τ-only derivative (Table 4, expt 5 and 6). A representative chromosomal τ-only *dnaX*(GCA428GCG; AAA429AAG) mutant, designated strain AB500, grew normally under laboratory conditions without detectable γ. Western blots detected no γ in whole-cell extracts of this strain (Fig. 4, lanes 1 and 2).

Purification of DNA Pol III* from the strain lacking γ. Pol III* contains all of the holoenzyme components except β (34, 56). An attempt to isolate Pol III* from the strain lacking γ enriched a preparation which, in the cell lysate supernatant, lacked γ. Although the purification procedure included the protease inhibitor phenylmethylsulfonyl fluoride in all

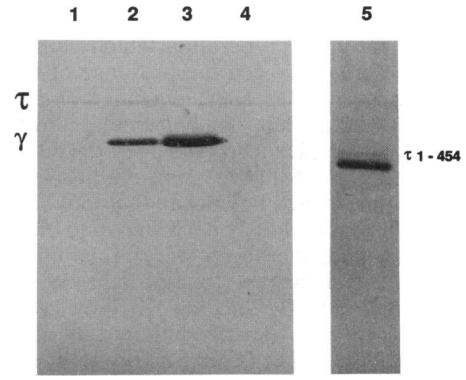

FIG. 3. Western blot of τ and γ from C600 strains containing plasmid-borne wild-type and γ-only *dnaX* alleles. Lanes: 1, C600 with no plasmid; 2, *dnaX*$^+$ pAB47; 3, *dnaX*(*Δ455-653*) pAB48; 4, pUC19; 5, pAB48, electrophoresed for a longer time to demonstrate τ1-454. All lanes were loaded with 5 μg of protein, except for lane 5, which received 7 μg of protein.

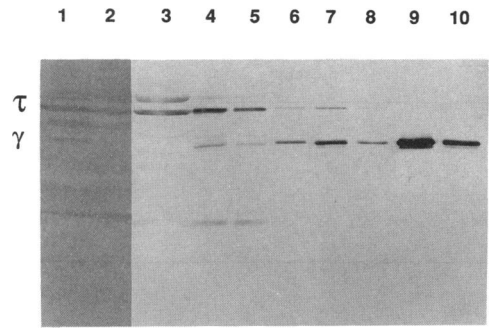

FIG. 4. Western blot of wild-type and τ-only whole-cell extracts (lanes 1 and 2, respectively) and DNA Pol III* preparations from a strain which produces τ only (AB500) (lanes 3 to 5 and 7 to 10). Lanes contain the following: 1 (*dnaX*$^+$ strain AB501) and 2 [τ-only *dnaX*(GCA428GCG; AAA429AAG) strain AB500], 200 μg of protein; 3, fraction I (cell lysate supernatant), 200 μg; 4 and 5, fraction II (dissolved ammonium sulfate precipitate), 19 and 3.8 μg, respectively; 6, purified τ and γ proteins, 4.8 and 7.0 ng, respectively; 7 and 8, fraction III (heparin-agarose column fraction), 450 and 180 ng, respectively; lanes 9 and 10, fraction V (ATP-agarose column fraction), 480 and 120 ng, respectively.

solutions, τ became unstable as Pol III* was purified. As purification proceeded, τ disappeared (Fig. 4). Apparently, τ was proteolyzed, because as τ disappeared, a new γ-like protein which migrated near the position of γ appeared. After ammonium sulfate precipitation, the preparation contained predominantly τ, although the γ-like protein was detectable (Fig. 4, lanes 4 and 5). After the heparin-agarose column, τ was only a minor fraction compared with the γ-like material (Fig. 4, lanes 7 and 8) and in the ATP-agarose column fractions, τ was undetectable and replaced by the γ-like protein (Fig. 4, lanes 9 and 10).

In the ATP-agarose column step of purification of wild-type Pol III*, τ and γ each bind the resin directly and other subunits are retained indirectly through their association with τ and γ. The polymerase core binds tightly to τ, but not to γ, indicating that the C-terminal portion of τ, missing in γ, is responsible for interaction with the polymerase (45). The Pol III core is retained on ATP-agarose through its strong interaction with τ. However, in this purification of Pol III* lacking γ, the core activity flowed through the ATP-agarose column because of loss of τ by proteolysis to form the γ-like protein. The Coomassie blue-stained SDS-polyacrylamide gel analysis of the final preparation which eluted from the ATP-agarose column revealed the presence of the δ, δ′, χ, and ψ subunits as well as a protein migrating near the γ position. The γ-like protein not only interacted with the γ antibody, but the complex also contained the activity which assembles β onto DNA for processive synthesis with the Pol III core, an activity which is γ dependent (36). The δ, δ′, χ, and ψ subunits have previously been shown to bind tightly to τ as well as to γ and, therefore, were probably bound to τ prior to its proteolysis to the γ-like protein (38, 59).

Constitution of Pol III* lacking γ. The entire Pol III holoenzyme has recently been constituted by using each of its individual subunits (38). Constitution studies with individual pure subunits have shown that (i) τ binds the core polymerase (45), (ii) τ binds a complex of δδ′ (38), and (iii) τ binds a complex of χψ (59). Hence, it seems likely that τ will assemble an eight-protein complex of Pol III* lacking γ. Indeed, an αεθτδδ′χψ complex was assembled upon mixing these subunits, as shown by the gel filtration sizing analysis

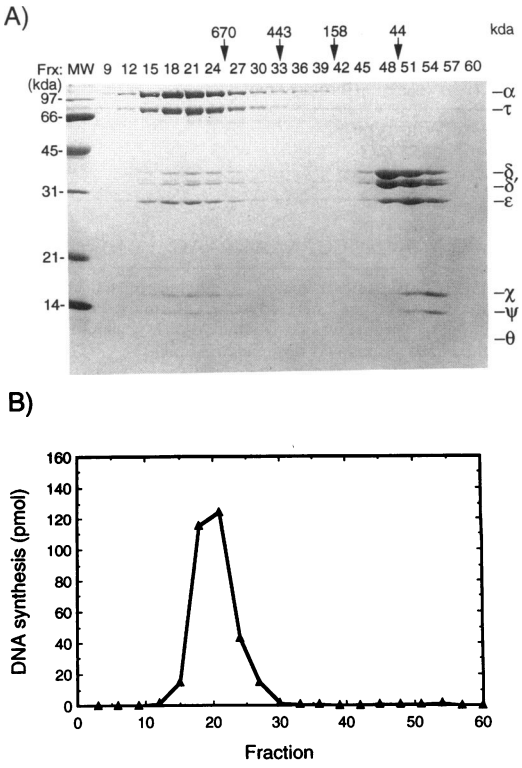

FIG. 5. Constitution of a Pol III* complex lacking γ. (A) Gel filtration. The α, ε, θ, τ, δ, δ′, χ, and ψ subunits were mixed, incubated, and then gel filtered (superose 6) as described in Materials and Methods. Column fractions (Frx) are indicated at the top of the gel. The first lane contained molecular weight (MW) standards and their masses are indicated to the left. The α, ε, θ, τ, δ, δ′, χ, and ψ subunits are identified to the right. The positions and molecular masses of protein size standards for the superose 6 gel filtration column are shown at the top of the gel. (B) Activity. Column fractions were assayed in the reconstitution of a processive polymerase β as described in Materials and Methods.

of Fig. 5. The Coomassie blue-stained SDS-polyacrylamide gel analysis of the gel filtration column fractions (Fig. 5A) showed comigration of these eight subunits in fractions 15 to 24, indicating that they are bound together in one large complex. A molar excess of the δ, δ′, χ, ψ, ε, and θ were added relative to τ and α (equimolar), and the unbound excess of these subunits migrated in later fractions, consistent with the mobility of the δδ′, χψ, and εθ complexes observed in previous studies (39, 46, 59). The Pol III* lacking γ was active, in the presence of added β, in extension of a single primer around single-strand binding-protein-coated M13mp18 single-stranded DNA circle (Fig. 5B).

DISCUSSION

γ is dispensable. Several earlier experiments had pointed to a central role for γ in polymerization. Polymerizing activity on single-strand, circular templates could be restored to heated extracts of the *dnaX2016*(Ts) (G118D) mutant by a wild-type protein isolated from a *dnaX*$^+$ strain and identified as γ (16, 53). Moreover, reconstitution of Pol III systems from purified proteins for polymerization of strands complementary to several natural, single-strand, circular templates with or without added primers always

required γ (53). γ was also required when polymerization on primed single strands was catalyzed by DNA Pol II (3, 53).

However, the γ subunit could be eliminated in vivo under typical laboratory growth conditions. The only obvious result of γ absence was that τ itself was proteolyzed during purification of the DNA Pol III* complex lacking γ. That is, the Pol III* complex was formed in the absence of γ, but its structure was such that τ apparently became sensitive to proteolysis in vitro since all previous preparations of Pol III* from wild-type strains contained τ (46a). E. Boye (3a) has determined that the τ-only mutant, growing in Luria broth plus glucose, is indistinguishable from the wild type in cell size, DNA content per cell, and cell cycle timing of replication initiation. Thus, γ is not required for normal progression of replication forks.

This demonstration that γ is dispensable in vivo is corroborated by in vitro complementation results of Maki and Kornberg (26). They found that heated extracts of *dnaX2016*(Ts) (G118D) mutant, inactive even at 30°C in the conversion of primed single-stranded circles to replicative form II, were complemented with equal specific activity by either τ or γ. However, heated extracts of the *dnaX36*(Ts) (E601K) mutant, which produces τ altered in residue 601, which were more thermolabile than the wild type, could be complemented only by added τ. This complementation by τ also required addition of core, suggesting that the presence of defective τ increased thermal lability of core as well (26).

The dispensability of γ is not too surprising given that τ contains all of the γ sequence, except for one residue, and can perform all the known biochemical functions of γ. Specifically, τ substitutes for γ in binding the δ, δ′, χ, and ψ subunits (38, 59) and is active with δ (and δ′) in placing β onto primed DNA (36). Furthermore, a Pol III* complex lacking γ can be reconstituted upon mixing τ with the α, ε, θ, δ, δ′, χ, and ψ subunits. It seems likely that this Pol III* lacking γ was present also in the τ-only strain before the cells were lysed, even though purification of the Pol III* lacking γ resulted in proteolysis of τ to a γ-like polypeptide. This interpretation is based on the fact that the δ, δ′, χ, and ψ subunits were present in the final preparation. At this time we cannot, however, rule out the possibility that in a mutant growing without γ, τ may be proteolyzed to a γ-like protein at a level below the detectability of the Western blot.

τ is essential. Several lines of evidence suggest that τ, the full-length product of *dnaX* translation, is essential. First, the conditional lethal *dnaX36*(Ts) mutation altered codon 601, which is located downstream of γ sequences in the τ-specific region of *dnaX*. Second, a partial diploid strain carrying the chromosomal *36*(Ts) allele could not be complemented by an allele which produces only γ. Third, the wild-type chromosomal gene could not be replaced by a γ-only allele (unless another copy of the wild-type gene was provided in *trans*). The essential nature of τ may seem somewhat surprising because earlier evidence for direct involvement in polymerization was not dramatic. The *dnaX36*(Ts) mutation altered codon 601, unique to τ, and, although it might be imagined that the mutant polymerization gene would result in rapid inhibition of polymerization at high temperature, this mutant stopped DNA synthesis only gradually, even at 44°C. Moreover, the temperature-sensitive phenotype could be observed only when the culture medium contained no (added) NaCl (14). τ, although observed in earlier Pol III* holoenzyme preparations (34), was not assigned as a holoenzyme component until 1982 after its identification as a subunit of DNA Pol III′ (αεθτ) (32). τ was not uniformly required, however, for polymerization of primed, natural single-strand circle complements. For example, McHenry (32) showed that Pol III′ (αεθ core plus τ) polymerized on primed fd DNA (when 5 mM spermidine was present) but was inert when primed G4 DNA was the template. Moreover, highly processive replication could be catalyzed by mixture of the nine subunits without τ. Maki and Kornberg (28) achieved highly processive conversion of M13G*ori*1 single strands to replicative form II by combining core (αεθ), β, and γ complexes (γδδ′χψ). While not essential, including τ with the core, β, and γ complex mixture increased total DNA synthesis (28). Thus, the present genetic study reveals an essential function or functions for τ which had not been detected in biochemical studies and suggests that a more relevant biochemical assay is needed.

What is the essential function(s) in vivo of τ which cannot be substituted by γ? It has previously been suggested that τ could be specific for lagging-strand synthesis. Wu et al. (58) demonstrated functional asymmetry in a coupled leading- and lagging-strand in vitro polymerization system which produced equivalent amounts of leading and lagging strands. Under standard reaction conditions, τ was not required. However, when ionic strength was increased, presumably destabilizing the replication fork, τ stimulated the reaction threefold, but only when lagging-strand synthesis was coupled to leading-strand synthesis. Thus, in this system, τ functioned only for lagging-strand synthesis, and, inasmuch as it was not required to initiate the formation of Okazaki pieces, it was proposed to accelerate movement of core from the 3′ end of completed Okazaki pieces to the 3′ end of new primers.

It has also been suggested that τ is specific for leading-strand synthesis. This was suggested by the demonstration that, although processive synthesis could be achieved by mixing purified core, β, and γ complex (27), further addition of τ stabilized initiation complexes and elongation complexes and boosted total synthesis (28). τ also averted pausing of holoenzyme at hairpinlike or double-stranded structures within the template. It was proposed, therefore, that, in the asymmetric holoenzyme dimer (17, 29, 43), the more stable τ half is responsible for leading-strand synthesis (28). τ has also been shown, by Studwell and O'Donnell, to stabilize initiation complexes (cited in reference 36).

A third unique function might be the τ ATP- and dATPase activity. Although a nucleotide binding site, defined by amino acid sequence analysis, is present in both τ and γ (62) and both bind ATP (1, 50), only τ hydrolyzes ATP at a significant level (23, 50). This single-strand DNA-dependent hydrolysis is weak, about 5 to 10 molecules of ATP per molecule of τ (50) (or τ-LacZ fusion protein [23]) per min. Although γ is not an ATPase (50), a DNA-dependent ATPase activity is present in the γ complex (γδδ′χψ) (10, 39) and in the two-protein γδ complex (O'Donnell et al., cited in reference 36).

A fourth essential unique function for τ could be interaction with δ′. Whereas γδ, τδ, or τδ′ can substitute for the γ complex in clamping β to primed templates and in promoting recycling (36), γδ′ could not. The fact that γδ′ could not effect this transfer might mean that an essential τ-δ′ interaction occurs in vivo.

A fifth possibility might be maintenance of core-γ complex interaction by binding to both. Although preinitiation complexes assemble in vitro in the absence of core, it is possible that, in vivo, lagging-strand initiation and polymerization are coupled.

The sixth and perhaps most important possibility is the ability of τ, but not γ, to bind directly to the polymerase (32,

45). The τ subunit dimerizes the core in this interaction by virtue of τ itself being a dimer in its native state (45). It is hypothesized that replicative polymerases act in pairs for simultaneous and coordinated replication of both the leading and lagging strands (17, 43). Hence, in *E. coli* lacking τ, this coordination would be lost. Inasmuch as cells lacking τ are not viable, it seems possible that coordination of leading and lagging strands may be essential for the duplication of the bacterial chromosome.

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