The DNA Replication Machine of a Gram-positive Organism*

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This report outlines the protein requirements and subunit organization of the DNA replication apparatus of *Streptococcus pyogenes*, a Gram-positive organism. Five proteins coordinate their actions to achieve rapid and processive DNA synthesis. These proteins are: the PolC DNA polymerase, τ, δ, δ', and β. *S. pyogenes* dnaX encodes only the full-length τ, unlike the *Escherichia coli* system in which dnaX encodes two proteins, τ and γ. The *S. pyogenes* τ binds PolC, but the interaction is not as firm as the corresponding interaction in *E. coli*, underlying the inability to purify a PolC holoenzyme from Gram-positive cells. The τ also binds the δ and δ’ subunits to form a τδδ’ “clamp loader.” PolC can assemble with τδδ’ to form a PolC-τδδ’ complex. After PolC-τδδ’ clamps β to a primed site, it extends DNA 700 nucleotides/second in a highly processive fashion. Gram-positive cells contain a second DNA polymerase, encoded by dnaE, that has homology to the *E. coli* α subunit of *E. coli* DNA polymerase III. We show here that the *S. pyogenes* DnaE polymerase also functions with the β clamp.

The replicative DNA polymerase III of Gram-positive cells, referred to herein as PolC, contains both DNA polymerase and proofreading 3'-5' exonuclease activities in one polypeptide chain (1–5). In contrast, the corresponding polymerase and 3'-5' exonuclease activities of the *E. coli* DNA polymerase III are contained on separate subunits, α (130 kDa, dnaE) and ε (28 kDa, dnaQ), respectively (6, 7). PolC has been purified from several different Gram-positive cells, and in each case the final preparation contains only a single subunit (2–5). In contrast, the replicative DNA polymerase III holoenzyme of *E. coli*, a Gram-negative cell, purifies as a particle consisting of ten different subunits, (1, 8). These several “accessory proteins” endow *E. coli* DNA polymerase III holoenzyme with special properties that are important to the task of rapidly replicating a long chromosome. Thus, they utilize ATP to clamp the polymerase tightly to DNA, endowing the polymerase with exceptionally high speed (−1 kb/s) and processivity (~50 kb) (9, 10). Cell division times of Gram-negative and Gram-positive cells are approximately similar, as are the sizes of their chromosomes. Therefore, it seems reasonable to expect that the PolC of Gram-positive cells also functions with accessory proteins that provide it with high efficiency in synthesis, like the *E. coli* DNA polymerase III holoenzyme.

The identification of Gram-positive PolC accessory factors is the subject of this report. The evolutionary split between Gram-positive and Gram-negative bacteria occurred over one billion years ago, consistent with the several important structural differences in their replicative DNA polymerases described above. The accessory proteins of these two cell types may also be expected to exhibit several differences. Nevertheless, the replication mechanisms of *E. coli* have generally served as a useful guide to studies in other systems (11, 12) and therefore a brief description of the structure and function of the accessory factors within *E. coli* DNA polymerase III holoenzyme follows.

*E. coli* DNA polymerase III can be purified in a variety of multisubunit forms of increasing complexity, the largest of which is the holoenzyme containing ten different subunits (reviewed in Ref. 13). The smallest subassembly, called DNA polymerase III core, is a heterotrimer consisting of each of α (DNA polymerase), ε (3'-5' exonuclease), and θ (14, 15). The 4-subunit DNA polymerase III* consists of two Pol III cores attached through a τ dimer bridge (16, 17). The two core polymerases perform concurrent synthesis of leading and lagging strands (18, 19). The τ subunit is the full-length product of dnaX; a translational frameshift generates the γ subunit (20–22). The γ subunit (47.5 kDa) is essentially the N-terminal two thirds of τ (71.1 kDa), as the frameshift results in only one unique C-terminal residue before arriving at a stop codon. The γ subunit associates with four smaller subunits: δ (38.7 kDa, holA), δ' (36.9 kDa, holB), χ (16.6 kDa, holC), and ψ (15.2 kDa, holD) (23–26). This 5-subunit “γ complex” associates with Pol III through a τ to γ contact to yield the 9-subunit Pol III* ((αεθδτ2 γδδ' χ1 ψ1) (27). The 10th subunit, called β (40 kDa), is a crescent-shaped molecule that dimerizes to form a protein ring (28). The β ring encircles duplex DNA and slides freely along it (29). The β sliding clamp binds Pol III* (or Pol III core) through the α subunit, serving as a mobile tether to hold the polymerase to DNA, being passively pulled along in back of the polymerase as nucleotides are incorporated (29). This action provides the polymerase with rapid speed and high processivity.

The β clamp does not assemble onto DNA by itself. For this it requires assistance of the γ complex. The γ complex couples ATP hydrolysis to open and close the β ring, assembling it around duplex DNA at a primed template junction (23, 30). Only the γ, δ, and δ’ subunits of the γ complex are required for clamp loading action (31). The δ subunit is the actual ring opener; it alone binds β and opens the dimer (32–34). Although the isolated δ subunit can remove β rings that have previously been assembled on DNA, δ cannot assemble β onto DNA by itself (33). The more complex process of assembling β onto DNA requires the coordinated actions of all three subunits, γδδ’,
with β and DNA. The γ subunit is the motor as it is the only subunit of the clamp loader machine that interacts with ATP (35). The δ subunit binds both γ and δ and coordinates changes in γ due to ATP binding, with access of δ to the β ring (26). In the ATP-bound state, δ is presented for action with β allowing the β ring to open (32). Upon binding a primed template, two ATP are hydrolyzed and the δ to β contact is severed, causing the release of the γ complex and allowing the ring to snap shut around the DNA (36, 37).

The strategy of using a sliding clamp and a clamp loader complex is conserved in eukaryotes and archaeabacteria and is even used by the T4 phage. In eukaryotes, the proliferating cell nuclear antigen homotrimer has essentially the same ring shape as *Escherichia coli* β, and the 5-subunit replication factor C acts as a clamp loader, coupling ATP hydrolisis to assembly of the proliferating cell nuclear antigen ring onto DNA (38, 39). The five replication factor C subunits are different proteins but are homologous to one another (in contrast, only the γ and δ subunits of the γ complex are homologous in sequence) (40).

PolC of Gram-positive cells almost certainly functions in the context of a sliding clamp, as early studies showed it was stimulated by accessory proteins of the *E. coli* system (3). Perusal of genome sequences of a wide variety of eubacterial organisms reveals that they contain homologues of the β clamp and two (γ and δ) of the five subunits of the γ complex. The γ and δ subunits are not sufficient for clamp loading action in the *E. coli* system; δ is also required (31). However, in some organisms only two subunits are sufficient to form a functional clamp loader as in archaea (42) and in phage T4 (43). Further, the dnaX gene of Gram-positive cells (i.e. *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) does not show an obvious frameshifting signal sequence. The full-length product of these dnaX genes (i.e. the τ subunit) of Gram-positive cells predicts a protein that has a mass that is less than that of *E. coli* τ but greater than γ. Does this intermediate length τ bind PolC? Finally, Gram-positive genomes contain a second essential polymerase gene, dnaE, that is also homologous to PolC and to *E. coli* α (44). Does this DnaE polymerase function with the β clamp, perhaps in chromosomal replication? The *S. pyogenes* DnaE polymerase (119 kDa) is similar in size to *E. coli* α (130 kDa), and it lacks the 3'-5' exonuclease domain, like *E. coli* α. In fact, PolC and the DnaE polymerase are more homologous to *E. coli* α than they are to one another.

This report examines the replication apparatus of *S. pyogenes*, a Gram-positive organism. The *S. pyogenes* PolC, DnaE polymerase, τ, δ, and β were expressed and purified. The τ and δ were insufficient for assembly of β onto DNA. We then identified *S. pyogenes* holA encoding δ; it is the least conserved of the subunits. The *S. pyogenes* δ, τ, δ, and τ form a complex, and the τδδ′ complex is functional in loading the *S. pyogenes* τ clamp onto DNA. Hence, three subunits are required for clamp loading, similar to the *E. coli* system. Although *S. pyogenes* τ is smaller than *E. coli* τ, the *S. pyogenes* τ subunit binds to PolC, forming a PolC-τδδ′ complex. However, the interaction between τ and PolC is not as strong as the connection between *E. coli* α and τ, thereby explaining why PolC is purified as a single subunit instead of as a PolC holoenzyme. *S. pyogenes* PolC-τδδ′ functions with β to produce a holoenzyme with comparable speed (~700 nucleotides/s) and processivity to the *E. coli* DNA polymerase III holoenzyme. The *S. pyogenes* DnaE polymerase is also stimulated by β, but its maximum speed is ~60 nucleotides/s making it less certain to be involved in bulk DNA synthesis at a moving replication fork.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radioactive nucleotides were from NEN Life Science Products; unlabeled nucleotides were from Pharmacia Upjohn. DNA oligonucleotides were synthesized by Life Technologies, Inc. The pET polymerase expression vectors and BL21 (DE3) *E. coli* were from Novagen. DNA modification enzymes were obtained from New England Biolabs. M13 gene 32 was from Dr. Susan Taylor (University of California, San Diego). Group C Streptococcus phage-associated lysin (46) was generously gift of Dr. Vincent Fischetti (The Rockefeller University).

**DNA—** *S. pyogenes* genomic DNA was isolated as described previously (47) from *S. pyogenes* (Lancfield group A, type 6) strain D471 propagated on THY media (1 liter of medium contained: 30 g of Todd-Hewitt medium, 2 g of yeast extract). M13mp18 ssDNA was purified from phage isolated by two successive bandings in cesium chloride gradients as described (48). M13mp18 ssDNA was primed with either a DNA 30-mer (map position 6817–6846) or DNA 90-mer (map position 4643–6694) as described (49). Circular plasmid DNA that was nicked once was prepared by treating plBluescript plasmid DNA (400 μg) with 35 units of *M. luteus* gene 32 and 20 μg of PolC (300 fmol), and 140 ng of PolC-τδδ′ complex or 100 ng of *E. coli* Pol IIIα in 25.5 mM of replication buffer (50 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 8 mM MgCl₂, 40 μg/ml BSA, 4% glycerol, 1 mM ATP, 60 μM each dCTP, dGTP, dTTP, and 20 μM [α-32P]dATP. An aliquot of the fraction to be assayed was added to the assay mixture on ice followed by incubation at 37 °C for 5 min. DNA synthesis was quantitated using DE81 paper as described (52).

**PolC Replication Assays—** Reaction conditions contained 70 ng (25 fmol) of 30-mer singly primed M13mp18 ssDNA, 0.82 μg of *S. pyogenes* or E. coli SSB, 45 ng of *S. pyogenes* or *E. coli* β (300 fmol), and 140 ng of PolC-τδδ′ complex or 100 ng of *E. coli* Pol IIIα in 25.5 mM of replication buffer (50 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 8 mM MgCl₂, 40 μg/ml BSA, and 60 μM each of dGTP and dCTP). For reactions that are less than 15 s, each time point is a separate reaction. Reactions were preincubated for 2 min at 30 °C to allow time for polymerase (PolC-τδδ′) and β to assemble at the primer terminus, and then DNA synthesis was initiated upon the addition of 1.5 μl of 1.5 mM dATP and 0.5 mM [α-32P]dATP yielding final concentrations of 60 and 20 μM, respectively. Reactions were quenched with an equal volume (25 μl) of a solution of 1% SDS and 40 mM EDTA. One-half of the quenched reaction was analyzed for total DNA synthesis using DE81 filter paper as described above, and the other half was analyzed for agarose gel electrophoresis.

Analysis of subunit mixtures were performed by first preincubating protein mixtures for 30 min at 15 °C as follows: 50 μg natural PolC, 10 μg/ml of τ, δ, or δ′. One microliter of preincubated mixture was added to 24 μl of a replication reaction containing 70 ng (25 fmol) of singly primed M13mp18 ssDNA, 43 ng of *S. pyogenes* β, and 0.82 μg of *S. pyogenes* SSB (when indicated) in replication buffer. Reactions were preincubated for 2 min at 37 °C, and then DNA synthesis was initiated upon the addition of 1.5 μl of 1.5 mM dATP and 0.5 mM [α-32P]dATP as described above, and the reaction was quenched after 15 s. One-half of the quenched reaction was analyzed in a 0.8% neutral agarose gel.

**DnaE Replication Assays—** Reaction conditions contained 70 ng (25 fmol) of 30-mer singly primed M13mp18 ssDNA, 0.82 μg of *S. pyogenes* or *E. coli* SSB, and 3.3–300 ng of DnaE (25 fmol to 2.3 pmol) in 23.5 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 40 μg/ml BSA, 2 mM ATP, 8 mM MgCl₂, and 60 μM each of dCTP and dGTP. Reactions performed to test for β stimulation of DnaE polymerase activity included 43.3 ng of β and 10 ng of τδδ′ protein complex. Reactions were preincubated for 3 min at 37 °C to allow time for polymerase and β clamp to assemble at the primer terminus, and then NaCl was added to the reaction to a final concentration of 40 mM for preincubation for another 3 min at 37 °C. DNA synthesis was initiated upon the addition of 1.5 μl of 1.5 mM ATP, 0.5 mM [α-32P]dATP (specific activity 2000–4000 cpm/mmol). Aliquots of 25 μl were removed at the indicated times and quenched with an equal volume (25 μl) of 1% SDS, 40 mM EDTA. One-half of the quenched reaction was analyzed for total deoxyribonucleotide incorporation using DE81 filter paper, and the other half was analyzed on a 0.8% neutral agarose gel.

**Template Challenge Assay—** The PolC holoenzyme was first clamped
onto the primed M13mp18 template in a preincubation containing 700 ng of 30-mer primed M13mp18 ssDNA, 7.5 μg of E. coli SSBI, 0.5 mM ATP, 120 μM each dCTP and dGTP, 60 ng of β, and 500 ng of PolC-αβδ (purified by gel filtration) in 125 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 80 mM MgCl₂, and 0.1 mM MgO. Preincubation at 37 °C (125 μl) was added that contained 5.8 μg of M13Gori1 ssDNA, 30 μg of E. coli SSBI, 0.5 mM ATP, 120 μM dATP, and 60 μM [α-32P]dTTP (specific activity 2000–4000 cpm/μmol) in 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 80 mM MgCl₂, and 40 μg/ml BSA. Aliquots of 50 μl were removed and quenched at the indicated times upon the addition of an equal volume of 10% glycerol, 0.5 mM EDTA and 2 mM DTT containing 100 μM NaCl. Protein column equilibrated with buffer A (20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 80 mM MgCl₂, and 0.4 mg of dTTP in a volume of 200 μl) and a mixture of (0.8 mg), δ (0.4 mg), and δ (0.45 mg) in a volume of 200 μl.

Protein standards (Bio-Rad and Sigma) were mixtures of 50 μg each in 100 μl of buffer A. Standards were bovine thyroglobulin (670 kDa, 85.0 Å), horse spleen apoferritin (440 kDa, 59.5 Å), β-amylose (200 kDa, 77.0 Å), bovine serum albumin (66 kDa, 44.9 Å), chicken ovalbumin (44 kDa, 27.5 Å, 3.6 S), and horse myoglobin (17 kDa, 19.0 Å, 2.0 S). To measure the Stokes radius of each S. pyogenes protein and subunit protein and complex, the K_{av} was calculated using the equation: K_{av} = (V_e - V_i) / (V_e - V_r), where V_e is the observed elution volume, V_i is the included volume, and V_r is the exclusion volume. V_e for the Superose 6 column is 24 ml, and the V_r, determined using M13mp18 ssDNA saturated with SSB (25 Mda), was 6.95 ml. The K_{av} was then plotted versus the known Stokes radii of the protein standards. The Stokes radius of S. pyogenes proteins was determined from the protein standards plot of K_{av} versus Stokes radius.

**Subunit Ratios by Gel Scan**—Protein concentrations were determined by absorbance at 280 nm using extinction coefficients calculated from published Trp and Tryptophan content using the equation ε_{280} = Trp (5690 m⁻¹ cm⁻¹) + Trypt (1280 m⁻¹ cm⁻¹). Extinction coefficients for 280 nm are as follows: PolC (109,520 m⁻¹ cm⁻¹), DnaE (99,420 cm⁻¹), δ (30,580 m⁻¹ cm⁻¹), ε (16,640 m⁻¹ cm⁻¹), θ (24,180 cm⁻¹), and β (5120 m⁻¹ cm⁻¹), SSB (16,500 cm⁻¹ cm⁻¹). DTT was removed from protein preparations by MonoQ chromatography of each protein prior to absorbance measurements in 8 μl vials. The absorbance spectrum was determined from 250 to 350 nm to ensure that DTT and DNA were removed. An equimolar mixture of PolC, δ, and β was used as standards in a 10% SDS-polyacrylamide gel. Amounts of standards loaded in gel lanes were (are for each individual subunit): 0.25, 0.5, 1, 2.5, 5, and 10 μg. Amounts of protein complex were: PolC-αβδ (1, 2.5, and 5 μg), αβδ (2.5 μg, 5 μg), and PolC-δ (2.5 μg, and 10 μg). Gels were stained with Coomassie Blue and then scanned using a Molecular Dynamics SI Densitometer.

**Glycerol Gradient Sedimentation**—Sedimentation analysis of PolC, δ, β, and δ mixtures of e, δ', δ'', δ''', PolC-αβδ, and PolC-αβδ were performed using 11.9 ml of 10–30% glycerol gradients in sedimentation buffer (20 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.5 mM EDTA, 100 μM NaCl). Either single proteins or protein mixtures (50 μg each PolC, DnaE, and/or δ) were incubated at 37 °C for 15 min in a volume of 200 μl of sedimentation buffer and then layered onto separate gradients. Protein standards (50 μg each in 100 μl of sedimentation buffer) were layered onto a separate gradient. Gradients were loaded into a Sorvall TH641 rotor and centrifuged at 40,000 rpm for 35 h at 4 °C. Fractions of 170 μl were collected from the bottom of the tube and analyzed on a 12% SDS-polyacrylamide gel (20μl lane) stained with Coomassie Blue.

**Gel Analysis**—Analysis of interactions among δ, δ', and δ'' by SDS-PAGE analysis, which forms the basis for pooling column fractions. Ammonium sulfate was added to the supernatant of the cell lysate to
pellet was resuspended in 50 ml of buffer A and dialyzed against buffer fate, and the precipitate was collected by centrifugation. The protein fractions 39–51 were precipitated with 0.226 g/ml of ammonium sulfate (0.3 g/ml) was added to the clarified cell lysate. The resulting pellet was resuspended and dialyzed against buffer A containing 50 mM NaCl and then loaded onto a 25-ml epoxy-activated agarose column (Bio-Rad). Protein was eluted with a linear 1.2-liter gradient of 50–500 mM NaCl in buffer A, 80 fractions were collected. Fractions 10–40 were pooled (164 mg), dialyzed against buffer A containing 50 mM NaCl, and then loaded onto a 25-ml epoxy-activated heparin-agarose column, and then eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed, each against buffer A, and applied to an 8-ml MonoQ column. The column was eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed against buffer A, and then aliquoted and stored at –80 °C.

Purification of \( S. pyogenes \) \( \beta \) Encoded by \( polC \)—The \( S. pyogenes \) \( polC \) was identified by searching the \( S. pyogenes \) data base with the sequence of the \( E. coli \) \( \beta \) subunit. The \( S. pyogenes \) \( polC \) encodes a 291-residue protein of 33,506 Da. The \( polC \) gene was amplified by PCR using the following oligonucleotide primers. The upstream 33-mer (5′-GCC TAG GAT AAG GGA GGG CAT ATG ATT TAG CGC-3′) contains an Ndel site (underlined) and a downstream 44-mer (5′-CGG GGA AGT CTT TTA ACA AGC TTC GGA TCC CCA TAA CGA ATG CC-3′) containing a BamHI site (underlined). The PCR product was digested with Ndel and BamHI, purified, and ligated into the Ndel and BamHI sites of pET11a to produce pETSpolC.

The pETSpolB plasmid was transformed into the BL21(DE3)recA. Cells (24L) were grown, induced, and lysed as described above for purification of \( \beta \) subunit. Ammonium sulfate (0.3 g/ml) was added to the clarified cell lysate. The resulting protein pellet was resuspended and dialyzed against buffer A containing 50 mM NaCl. The dialyzed protein (300 mg) was applied to a 45-ml FFQ Sepharose column, and the protein was eluted with a 450-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions (16–30, 284 mg) were combined, dialyzed against buffer A containing 50 mM NaCl, and then loaded onto a 25-ml epoxy-activated heparin-agarose column, and then eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed, each against buffer A, and applied to an 8-ml MonoQ column. The column was eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed, each against buffer A, and then aliquoted and stored at –80 °C.

Purification of \( S. pyogenes \) \( \beta \) encoded by \( dnaN \)—A search of the \( S. pyogenes \) genome data base using the \( E. coli \) \( \beta \) subunit amino acid sequence resulted in a DNA sequence with a high scoring match. The \( S. pyogenes \) \( dnaN \) encodes a 369-residue protein of 40,248 Da. The \( dnaN \) gene was amplified by PCR using the following primers: the upstream 34-mer (5′-GGG TTG CAT ATG ATT CAA TTT TCA AAT TAA TCG C-3′) contains an Ncol site (underlined), and the downstream 35-mer (5′-TAT CAG CTC CTT GTA GCA GTA CCT TCT ATG TAG CCT CC-3′) contains a BamHI site (underlined). The PCR product was digested with Ncol and BamHI, purified, and ligated into the pET11b vector to produce pETSpdnAN.

The pETSpdnAN plasmid was transformed into the \( E. coli \) strain BL21(DE3)recA. Cells (24L) were grown, induced, and lysed as described above for purification of \( \beta \) subunit. Ammonium sulfate (0.3 g/ml) was added to the clarified cell lysate. The resulting protein pellet was resuspended and dialyzed against buffer A containing 50 mM NaCl. The dialyzed protein (300 mg) was applied to a 45-ml FFQ Sepharose column, and the protein was eluted with a 450-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions (16–30, 284 mg) were combined, dialyzed against buffer A containing 50 mM NaCl, and then loaded onto a 25-ml epoxy-activated heparin-agarose column, and then eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed, each against buffer A, and applied to an 8-ml MonoQ column. The column was eluted with a 80-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. The peak fractions were pooled (fractions 10–27, 160 mg), dialyzed, each against buffer A, and then aliquoted and stored at –80 °C.
Centrifugation and then resuspended in 5 ml of buffer A (10 mg/ml). Ten milligrams was dialyzed and applied to a 10-ml ssDNA-agarose column (Life Technologies, Inc.) and eluted with a 100-ml linear gradient of 50–500 mM NaCl in buffer A; 80 fractions were collected. Fractions 30–50 containing PolC (>95% pure) were pooled (4 mg) and stored at −80 °C.

Purification of the S. pyogenes gene encoding the reaction of the subunit yields genes encoding PolC and DnaE. The DNA gene encodes a 1034-residue protein of 118,953 Da. The DNA gene was amplified by PCR using an upstream (5′-GGG AAC AAT ACC ACG GAA CAT ATG GTT GCT CCA GGA TCC TCG CCG CCA CTG G-3′) containing an Nde I site (underlined) and a downstream (5′-CGA ATA GCA GCG TTC ATA CCA GGA TCC TTC ATG GAT CAT ATG ATT AAC AAT GTA GCA GTA CTA GGT GGC GGC GCG TCT-3′) containing a Bam HI site (underlined). The PCR product was digested with Nde I and BamHI, purified, ligated into pET11a to produce pETSpSSB.

The pETSpSSB plasmid was transformed into the E. coli strain BL21(DE3)recA. Cells (24 L) were grown, induced, and lysed as described above for purification of the subunit. The cell lysate was clarified by centrifugation at 13,000 rpm for 30 min, and SSB was significantly purified by sequential fractionation with ammonium sulfate in the following manner. Solid ammonium sulfate was added to the clarified lysate and the precipitate was collected by centrifugation at 13,000 rpm for 30 min. The resulting pellet was homogenized in buffer A containing 0.1 M NaCl and 0.24 g/ml ammonium sulfate using a Dounce homogenizer, and then the precipitate was collected by centrifugation. This procedure was repeated with buffer A containing 0.1 M NaCl and 0.24 g/ml ammonium sulfate, then buffer A containing 0.1 M NaCl and 0.15 g/ml ammonium sulfate, and finally buffer A containing 0.1 M NaCl and 0.13 g/ml ammonium sulfate. The final pellet was resuspended in buffer A containing 0.15 M NaCl and dialyzed against the same buffer. The resulting pellet was resuspended in buffer A and dialyzed against buffer A containing 500 mM NaCl. The dialysate (300 mg) was diluted to 0.15 M NaCl and then loaded onto a 20-ml MonoQ column and eluted with a 200-ml 0.15–0.5 M NaCl linear gradient in buffer A; 80 fractions were collected. The SSB protein eluted in the very beginning of the gradient. Fractions 16–30 were pooled (150 mg), diluted to 0.05 M NaCl, and loaded onto a 10-ml ssDNA-agarose column. The column was eluted with 100 ml of buffer A containing 0.1 M NaCl; 80 fractions were collected. The peak fractions (32–62) were pooled (120 mg), aliquoted, and stored at −80 °C.

RESULTS

Genes Encoding Subunits of the S. pyogenes Replicase—Genes encoding PolC, DnaE, and SSB were easily identified by searching the S. pyogenes genome using the E. coli dnaA, dnaX, holB, dnaN, and sib genes. Each gene was amplified, cloned into the pET vector, expressed, and purified as described under “Experimental Procedures.” The Coomasie Blue-stained SDS-polyacrylamide gel of the final preparations is shown in Fig. 1. Expression of S. pyogenes dnaX in E. coli produced only one protein. The S. pyogenes DnaX protein migrates in an SDS-polyacrylamide gel in a position consistent with the full-length 62-kDa protein, which we refer to herein as θ.

A characteristic of the E. coli replicative Pol III holoenzyme is its high efficiency of synthesis on a very long primed ssDNA template coated with SSB. For example, once the E. coli holoenzyme has clamped onto a singly primed bacteriophage
**Fig. 4. Constitution of the \( \tau \delta \delta' \) clamp loader.** Subunits were mixed, incubated, and then analyzed by gel filtration as described under "Experimental Procedures." A, \( \tau + \delta + \delta' \); B, \( \tau + \delta' \); C, \( \tau + \delta \); D, \( \delta' \); E, \( \delta \); F, \( \delta + \delta' \). Fraction numbers are indicated above the gels, and individual subunits are identified to the right of each gel. The elution position of size standards are indicated at the bottom of the gels in C and F. S. pyogenes \( \delta' \) often migrates as a doublet in SDS-polyacrylamide gels, similar to the behavior of E. coli \( \delta' \).

A study of protein-protein interaction among the S. pyogenes \( \delta, \tau, \) and \( \delta' \) subunits is shown in Fig. 4A is a gel filtration analysis of a mixture of \( \tau, \delta, \) and \( \delta' \); the three subunits co-migrate in fractions 30–34 (\( \delta' \) appears as a doublet in SDS gels even though it is an intact protein, as demonstrated earlier in the E. coli system (24)). Densitometry analysis of the Coomassie Blue-stained gel was performed, and a standard curve was used to analyze the gel (Table I). The results indicate a ratio of \( \tau: \delta: \delta' \) of 4:1:1, indicating that a \( \tau \) tetramer binds one each of \( \delta \) and \( \delta' \). This stoichiometry is the same as that reported for the analogous E. coli subunits by Dallmann and McHenry (41) and somewhat greater than the estimates of 2–3 \( \gamma \)one each of \( \delta \) and \( \delta' \) reported by Onrust et al. (26) and Maki and Kornberg (23). Analysis of mixtures of \( \tau, \delta, \) and \( \delta' \) (B) and of \( \tau \) and \( \delta \) (C) does not demonstrate co-migration of subunits. Hence, both \( \delta \) and \( \delta' \) must be present together to detect a stable complex with \( \tau \). A mixture of \( \delta \) and \( \delta' \) subunits, on the other-hand, form a \( \delta \delta' \) complex (F) and co-elute earlier than either subunit alone (D and E). Densitometry analysis of the \( \delta \delta' \) complex (Table I) indicates that they form a \( \delta, \delta' \) complex. The Stokes radius of \( \tau, \delta, \delta' \) and the \( \delta \delta' \) complex was obtained upon comparison to standards of known Stokes radius (Table I). The S values of \( \tau, \delta, \delta' \) and \( \delta \delta' \) complex were also determined by glycerol gradient analysis compared with standards of known S values (Table I). These sizing techniques suffer from the fact that the shape of the protein influences its behavior in addition to its mass. The shape factor can be eliminated, and a molecular mass was estimated, by combining the Stokes radius and S value in the equation of Siegel and Monty (53). Combining the Stokes radius and S value in the equation of Siegel and Monty (53) yields
a native mass of 46 kDa for δ and 44 kDa for δ' within 15–30% of the mass calculated from their gene sequences, indicating that they are monomers (Table I), as is the case for E. coli δ and δ' (31). A similar analysis of the δδ' complex gives a mass of 87 kDa, consistent with a δ1δ'1 complex (calculated mass = 73 kDa), similar to the E. coli δ1δ'1 complex (31). Analysis of τ with either δ or δ' showed the peak of τ eluted in fractions 30–34 (τ alone elutes at this position, see Fig. 6). Comparison to protein standards of known Stokes radius yields a radius of 63 Å for τ (Table I). In a glycerol gradient analysis τ sedimented with an S value of 7.8. Combining these values in the Siegel and Monty (53) equation results in a native mass of 205 kDa suggesting a S value of 7.8. Combining these values in the Siegel and Monty equation results in a native mass of 205 kDa suggesting a S value of 7.8. Combining these values in the Siegel and Monty (53) equation results in a native mass of 205 kDa suggesting a S value of 7.8. Combining these values in the Siegel and Monty (53) equation results in a native mass of 205 kDa suggesting a S value of 7.8.

Constitution of a PolC-δδ' Complex—In the E. coli system, δ clamps that have been assembled onto circular DNA remain stably attached to the DNA during gel filtration. Because of the unique topological binding of the δ ring to DNA and its ability to slide, linearization of the DNA results in dissociation of δ from DNA by sliding off over the DNA ends (29). Next we examined S. pyogenes τ for ability to stably bind DNA yet freely slide along it.

We examined S. pyogenes δδ' for ability to load onto circular DNA in the experiment of Fig. 5A. The S. pyogenes δ ring was labeled with 32P by engineering a 6-residue C-terminal tail onto the protein, which is a recognition sequence for cAMP-dependent protein kinase. The 32P-δ was mixed with M13mp18 plasmid DNA that contained a single site-specific nick (using the gene protein II), and then δδ' was added. After 5 min at 37 °C the reaction was gel-filtered over a BioGel A15 m column. Protein not bound to DNA is included in this large pore resin (elutes in fractions 20–30), but 32P-δ bound to the large DNA elutes in the excluded fractions (fractions 12–16). The results, in Fig. 5A, show that 32P-δ comigrates with the DNA only in reactions containing δδ', demonstrating that δδ' is indeed an active clamp loader. The assembly reaction requires ATP (data not shown).

Demonstration that the 32P-δ clamps bind DNA topologically is shown in Fig. 5B. After assembly of 32P-δ onto DNA, the reaction was divided and one half was treated with EcoRI, which cuts the plasmid once; the other half was treated with EcoRI storage buffer. The reactions were then analyzed by gel filtration. The results show that linearization of the plasmid releases 32P-δ from DNA, and therefore S. pyogenes δ binds DNA topologically, as expected for a ring-shaped protein.

Constitution of a PolC-δδ' Complex—In the E. coli system, the τ subunit binds Pol III core to form the dimeric Pol III complex (16, 17). In Fig. 6, we examined the ability of S. pyogenes τ to bind PolC by gel filtration analysis. Analysis of τ alone showed that the peak of τ eluted in fractions 30–34. Gel filtration analysis of PolC, in Fig. 6A, showed it eluted in fractions 36–40, near the 158-kDa size marker, consistent with a monomeric state of PolC (164.5 kDa). The PolC of B. subtilis, another Gram-positive organism, is also reported to be monomeric (3). Glycerol gradient sedimentation analysis of PolC yielded an S value of 7.1 (Table I). Combination of the S value and 50 Å Stokes radius yields a mass of about 154 kDa, again consistent with a monomeric state of PolC (Table I).

Analysis of a mixture of PolC and τ showed that they comigrate and elute earlier than either protein alone, in fractions 28–32, demonstrating that they form a complex (Fig. 6C). However, a glycerol gradient analysis of PolC and τ showed no complex formation indicating that the PolC to τ interaction is not stable over the long time frame of a glycerol gradient sedimentation experiment (not shown).

The S. pyogenes τ is capable of binding δδ' and of binding PolC. Can it bind all three of these proteins at once to form a
4-subunit PolC-720 complex? The PolC, δ, and ε subunits were mixed and analyzed on a Superose 6 sizing column. The Coomassie Blue-stained SDS-polyacrylamide gel of the column fractions, shown in Fig. 7C, demonstrates that all four proteins comigrate (fractions 26–30) and elute earlier than either PolC or ε5 alone (see A and B), indicating that they form a large complex. In this experiment, PolC was added in a molar excess to τ, and therefore PolC elutes in two peaks, the first comigrates with τ, δ, and ε; the second is at approximately the position of PolC alone. The densitometric analysis of the PolC-ε5 complex, in fractions 24–30, indicates a stoichiometry of PolC-τδεε5 (Table I). Alternatively, the complex in solution contains four molecules of PolC, and it partially dissociates during gel filtration.

**Comparison of S. pyogenes PolC-ε5-β to E. coli Pol III Holoenzyme—**In the experiment of Fig. 8, the speed of the *S. pyogenes* holoenzyme was examined and compared with that of the *E. coli* Pol III holoenzyme. In Fig. 8A, *S. pyogenes* PolC-ε5 complex and β were incubated with singly primed M13mp18 ssDNA coated with *S. pyogenes* SSB. The reaction was preincubated for 5 min at 37 °C in the presence of ATP, dCTP, and dGTP allowing the assembly of β and PolC-ε5 on the DNA (see scheme in the Fig. 8). Under these conditions the primer cannot be extended for lack of dATP and dTTP. Hence, after the PolC-ε5-β complex assembles onto the DNA, it is constrained to “idle” at the primed site, alternately incorporating dCTP and dGTP and removing them via the 3′–5′-exonuclease activity. Extension is then initiated upon addition of the remaining two dNTPs (dATP and [α-32P]dTPP), and the reaction is quenched with SDS/EDTA at the indicated times (each time point is a separate reaction). DNA products are then analyzed in a native and an alkaline gel followed by autoradiography.

The results of the native gel, in Fig. 8A, demonstrate that *S. pyogenes* PolC-ε5-β forms full-length RFII product in ~10 s for a speed of about 720 nucleotides/s. Results of a similar experiment, using *E. coli* Pol III holoenzyme, are also shown in Fig. 8A. The *E. coli* Pol III holoenzyme completes the 7.2-kb primed template within 12 s for a synthetic rate comparable to the *S. pyogenes* system, at least within the experimental error for these rapid manual time points. The panels in Fig. 8B show the results of the alkaline gels. Comparison of each time point under 10 s confirms that the replicates of these two systems move at comparable rates, between 650 and 800 nucleotides/second.

Next, we examined the processivity of the *S. pyogenes* PolC holoenzyme. The challenge experiment of Fig. 9 examines the enzyme for ability to remain attached to an M13mp18 ssDNA-primed template the entire time that the primer is being extended around the circle. In this experiment, the PolC-ε5-τ complex and β clamp were assembled onto SSB-coated primed M13mp18 ssDNA in a preincubation, as described under “Experimental Procedures.” A shows PolC alone, B is τ alone, and C is the PolC-τ complex formed upon mixing PolC and τ. Fraction numbers are indicated at the top and bottom of the figure. The elution positions of protein standards are indicated at the bottom.

**Fig. 6. Constitution of S. pyogenes PolC-τ complex.** PolC and τ were analyzed alone, or as a mixture, by gel filtration as described under “Experimental Procedures.” A shows PolC alone, B is τ alone, and C is the PolC-τ complex formed upon mixing PolC and τ. Fraction numbers are indicated at the top and bottom of the figure. The elution positions of protein standards are indicated at the bottom.

shown that PolC was stimulated by partially purified preparations of *E. coli* DNA polymerase III subunits (3). These preparations, called elongation factors I and II, likely correspond to the β clamp and γ complex clamp loader (30). Presumably, *E. coli* γ complex assembled *E. coli* β onto DNA whereby *B. subtilis* PolC bound to it increasing its processivity. Functional exchange of these replication components from such diverse bacteria is quite astounding given the low level of sequence homology between them (β, 22%; PolC/α, 21%).

Now that we have all the components needed to reconstitute a rapid, processive replicase from a Gram-positive cell, we have more thoroughly examined the ability of proteins of one system to substitute for those of the other. In Fig. 10 each of the three *S. pyogenes* components, PolC, β, and the ε5′ clamp loader, were examined for the ability to substitute for the corresponding *E. coli* component using primed M13mp18 ssDNA as a substrate. A positive result is indicated by complete primer extension around the 7.2-kb template to form the nicked circular duplex RFII DNA. The PolC (*S. pyogenes*) and core polymerase (*E. coli*) are not capable of forming an RFII product on their own under the conditions used in this experiment. Thus, the RFII DNA product is only formed upon assembly of β onto DNA by the clamp loader and productive interaction between β and the polymerase. The M13mp18 ssDNA template was coated with *E. coli* SSB as we find that both systems function to form RFII products regardless of which SSB is used (data not shown). Reaction products were analyzed in a native agarose gel followed by autoradiography to visualize RFII DNA. The results, in Fig. 10, show that the PolC polymerase is functional with *E. coli* β provided the *E. coli* γ complex is also present, indicating a conserved interaction between PolC and the β ring. This result is consistent with the previous study of *B. subtilis* PolC and *E. coli* accessory factors (3). However, no other exchange experiments gave a positive result. The *S. pyogenes* ε5′ did not substitute for γ complex in this assay, indicating that *S. pyogenes* ε5′ does not assemble *E. coli* β onto DNA. Further,
E. coli core polymerase does not utilize the *S. pyogenes* β clamp assembled onto DNA using *S. pyogenes* τδδ' (28). Hence, *E. coli* β appears uniquely capable of functioning with both *E. coli* core and *S. pyogenes* PolC; *S. pyogenes* β only functions with *S. pyogenes* PolC. In experiments not shown here, we have studied individual τ, δ, and δ' subunits for the ability to substitute for one another in these two systems, but they do not.

**DnaE Polymerase Functions with the β Clamp**—The *S. pyogenes* DnaE polymerase is more homologous to *E. coli* α than *S. pyogenes* PolC, and thus it seems reasonable to expect that the DnaE polymerase may also function with the β clamp. Despite the homology, the DnaE polymerase is quite efficient on its own in extending a primer full circle around M13mp18 ssDNA in the presence of SSB. In contrast, the *E. coli* core polymerase is extremely inefficient in extending a primer on an SSB-coated M13mp18 ssDNA (53).

At a saturating concentration of DnaE polymerase, the time course of primer extension shows that it completes an M13mp18-primed ssDNA template within 2 min for a speed of at least 60 nucleotides/s (Fig. 11A). This rate of synthesis holds true for the highest amount of DnaE in the rightmost panel of Fig. 11A. As the DnaE concentration is decreased, a longer time is required to complete the circular template, indicating that the DnaE polymerase is not processive over the entire length of the M13mp18 template. If the DnaE polymerase were fully processive during synthesis of the 7.2-kb ssDNA circle, the product profile over time would be qualitatively similar at all concentrations of enzyme, but the overall intensity of the profile would be diminished. This particular experiment in Fig. 11A was performed in the absence of β but in the presence of τδδ'. We have repeated this experiment in the presence of β without τδδ', and in the absence of both β and τδδ', the results are similar to those shown in Fig. 11A.

In the presence of β and τδδ', DnaE polymerase is stimulated in synthesis at low concentration, indicating that β increases the processivity and/or speed of DnaE (Fig. 11). At higher concentrations of DnaE, the presence of βτδδ' has no effect on the rate of synthesis, and thus β does not increase the intrinsic speed of the enzyme (i.e., the last two panels of Fig. 11B). Hence, the effect of the β clamp on DnaE is primarily due to an increase in processivity. The profile of product length over time remains essentially unchanged at the different DnaE concentrations, and therefore the processivity of DnaE with β is at least equal to the 7.2-kb length of the M13mp18 substrate.

The DnaE sequence does not show homology to an exonuclease, implying that it may have no associated nuclease activity. The DnaE preparation was examined for the presence of a 3'-5'-exonuclease in panel C of Fig. 11. The DnaE and PolC polymerases were each incubated with a 5'-32P-labeled oligonucleotide, followed by analysis in a sequencing gel. The result showed no degradation of the oligonucleotide by DnaE. PolC is a known 3'-5'-exonuclease, and it digests the end-labeled oligonucleotide as expected.

Gram-positive PolC is known to be inhibited by the antibiotic hydroxyphenylazacytosine and its derivatives (54–57). In Fig. 11D, the PolC-τδδ', β, and DnaE were tested for inhibition of
The Stokes radius and S value of each subunit and the δβ' complex were determined from gel filtration and glycerol gradient analysis. Molecular masses were calculated from the Stokes radius and S value using the equation of Siegel and Monty (53). These calculations require the partial specific volume of each protein, which were calculated by summation of the partial specific volumes of the individual amino acids for PolC, τ, δ, and δ' (68). The native aggregation state of protein complexes was inferred from the ratio of subunits within them as determined from densitometric analysis of Coomassie Blue-stained gels compared to a standard curve constructed using known amounts of each protein in the same gel, each of which were quantitated by measurement of their absorbance at 280 nm and their known molar extinction coefficients. The molecular mass of each protein complex was calculated from the subunit stoichiometry, and the mass of each subunit was predicted from the gene sequences.

Table I

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DDiscussion

Gram-positive Cells Utilize PolC Holoenzyme—The E. coli Pol III* is a tightly associated particle that contains two core polymerases and one clamp loader, whereas PolC from Gram-positive cells is purified as a single subunit (2–5). This study on the S. pyogenes replication system demonstrates that S. pyogenes has several subunits that assemble with PolC into a holoenzyme and endow it with high speed and processivity comparable to the E. coli Pol III holoenzyme. The four main subunits in the E. coli system needed to provide speed and processivity to the core polymerase are the β clamp and the three subunits of the clamp loader, γ (or τ), δ, and δ'. This report shows that the Gram-positive replicase employs a similar strategy using the same four subunits. Genomic comparisons failed to recognize the δ subunit because of a low level of sequence conservation during evolution.

Why is PolC purified as a single subunit instead of a larger multiprotein complex containing accessory factors? Our studies of the S. pyogenes system indicate that the underlying basis for inability to purify a multisubunit PolC holoenzyme from Gram-positive cells is a relatively unstable interaction between τ and PolC. Although not shown here, we find that the PolC-τ complex does not remain associated during sedimentation in a glycerol gradient or during gel filtration under dilute conditions (i.e. 0.2 M). In contrast, the E. coli a-τ contact is stable to these procedures even at concentrations well below 100 mM. Most other replicases also lack a strong interaction between the polymerase and clamp loader. For example, the T4 polymerase (gene protein 43) is isolated as a single subunit, and the clamp loader (gene protein 44/62) is purified separately. This is also the case in the yeast and human systems where the DNA polymerase δ (2–4 subunits) is purified as a separate entity from the RF-C clamp loader. Presumably, these replicase components stably adhere, together with other replication proteins (e.g. helicase, primase, and SSB), at a moving replication fork.

Why Does S. pyogenes dnaX Not Encode a γ Subunit?—The dnaX genes of several Gram-negative cells produce two products; τ is the full-length species and γ is shorter, being produced by a translational frameshift. The presence of two dnaX products has been observed in the following enterobacteria: E. coli, Enterobacter aerogenes, Shigella flexneri, and Salmonella typhimurium (58). In each case, the smaller of the two subunits may be produced by translational frameshifting as the respective dnaX genes have consensus translational frameshift sites. The Gram-negative bacterium, Thermus thermophilus, also produces two proteins from the dnaX gene, although in this organism the two products derive from transcriptional slippage that generates a stop codon (59). In contrast, Western analysis of nonenterobacteria Gram-negative cells, Aeromonas hydrophila and Vibrio cholerae, indicates that only one protein is produced from the dnaX gene, consistent with lack of a discernible frameshift signal in their dnaX gene sequences (58).

There is no obvious translational frameshift signal in the S. pyogenes dnaX gene, and this report shows that expression of S. pyogenes dnaX in E. coli produces only τ, the full-length protein. Genes encoding subunits of the T4 and eukaryotic clamp loaders also produce only one protein each. Why do some organisms, like E. coli, go through so much trouble to produce a γ subunit in addition to τ?

The question of why γ is produced in some organisms will require further study, but we shall like to make a correlation, which may shed light on this subject. Of the five replication systems, S. pyogenes, E. coli, T4, yeast, and human, only E. coli produces both γ and τ from one gene, and only E. coli has a clamp loader that is tightly bound to the polymerase. The replication apparatus of all other organisms studied thus far lack this firm attachment between the clamp loader and polymerase, including S. pyogenes. Thus, their clamp loaders and polymerase purify as separate entities.

Having a clamp loader that is separate from the polymerase may be important to the replication process. Why would this be so? The reasoning is as follows. During discontinuous synthesis of the lagging strand, the polymerase must rapidly hop from a completed fragment to the RNA-primed start of the next fragment. In this hopping process, the polymerase leaves the clamp behind on the completed Okazaki fragment, and it hops to a new clamp that has been assembled on the next RNA primed site by the clamp loader (30, 45). The leftover clamps remain firmly attached to the DNA (18). Clamps are relatively abundant in cells and they accumulate on DNA as the replication machinery progresses forward (18). Indeed, in some systems, polymerase sliding clamps have been shown to have downstream uses, interacting with processing factors and even coordinating replication with transcription of some genes (60–62). However, Okazaki fragments far outnumber clamps, and synthesis on SSB-coated primed M13mp18 ssDNA by an hydroxyphenylaza-uracil derivative, TMAU. The PolC-τδδ'β' enzyme was prevented from forming the RFII product by TMAU. In contrast, the DnaE polymerase was not affected by TMAU in zyme was prevented from forming the RFII product by TMAU. i.e. conditions (or, dation in a glycerol gradient or during gel filtration under dilute gular strategy using the same four subunits. Genomic compari-
they must be recycled numerous times for synthesis of thousands of Okazaki fragments. How do the clamps recycle? Here is where having a separate clamp loader may be useful, as the clamp loader has been shown to be capable of unloading clamps in both the E. coli and human replication systems (33, 63, 64). Organisms in which the clamp loader is firmly attached to the polymerase may not be able to unload the clamps, as the clamp loader will be sequestered to sites of polymerase action where rapid and repeated clamp loading action is required (i.e. the replication fork). However, replicases that lack this firm attachment could utilize a clamp loader that is separate from the polymerase for clamp unloading action at sites distant from the fork.

In the above scenario, one may ask how E. coli cells unload clamps if the clamp loader is firmly attached to the polymerase. In vitro reconstitution studies show that the γ complex (γδβγδ) binds Pol III* (αεστγδ) through a γ to σ contact to form Pol III* (αεστγδγδ) and that this contact cannot be formed if the γ complex has already assembled before contact with σ has been established (27). Hence, production of γ may provide a mechanism for E. coli to produce some γ complex that remains separate from the holoenzyme particle for clamp unloading. Indeed, cell fractionation studies indicate that E. coli cells contain a variety of Pol III subassemblies, including γ complex and Pol III*. It should be noted that E. coli dnaX mutants that do not produce γ are viable, although production of some intracellular γ by proteolysis of τ could not be excluded (65). Despite these arguments, it is of course possible that γ is produced for some other function specific to E. coli and a few other Gram-negative cells.

The DnaE Polymerase—Gram-positive bacteria contain two DNA polymerases that have homology to E. coli α, one is PolC and the other is encoded by a gene encoding a protein that is homologous to E. coli α and β were assembled onto primed M13mp18 ssDNA (7.2 kb) in a preincubation. Following this, a 7-fold molar excess of primed M13Gori ssDNA (8.6 kb) was added, and DNA synthesis was initiated. Aliquots were removed at the indicated times and quenched, and products were analyzed on a native agarose gel as described under “Experimental Procedures.” Positions of M13mp18 RFII and M13Gori RFII duplex products are indicated to the left of the autoradiogram.

FIG. 9. S. pyogenes PolC holoenzyme is processive. Processivity was tested in a template challenge experiment. S. pyogenes PolC/αβγδ and β were assembled onto primed M13mp18 ssDNA (7.2 kb) in a preincubation. Following this, a 7-fold molar excess of primed M13Gori ssDNA (8.6 kb) was added, and DNA synthesis was initiated. Aliquots were removed at the indicated times and quenched, and products were analyzed on a native agarose gel as described under “Experimental Procedures.” Positions of M13mp18 RFII and M13Gori RFII duplex products are indicated to the left of the autoradiogram.

The homology of S. pyogenes DnaE polymerase to E. coli α suggests that DnaE may function with the β clamp like the α subunit of E. coli core and Pol III*. We find here that DnaE does indeed function with β. DnaE alone is a rather potent DNA polymerase that can extend a primer completely around an SSβ-coated primed M13mp18 ssDNA circle on its own with a speed of ~60 nucleotides/s. The β clamp increases the processivity of DnaE but does not appear to increase its intrinsic speed. In this regard, DnaE behaves similarly to E. coli DNA polymerase II. The processivity of DNA polymerase II is enhanced by β and γ complex (9, 67), but the intrinsic speed remains ~40 nucleotides/s (67).

Does DnaE polymerase participate in chromosomal replication? There is a report indicating that the dnaE gene is essential to Streptomyces coelicolor, a Gram-positive organism (44). The current study examined the DnaE polymerase for inhibition by TMAU, but no inhibition was observed. Hence, this drug would appear to target only PolC. The intrinsic speed of the DnaE polymerase is probably too slow to account for participation of DnaE in bulk DNA synthesis. However, it remains possible that the rate of synthesis by DnaE may be increased upon association with other replication proteins. Perhaps S. pyogenes even uses two different DNA polymerases for leading and lagging strand synthesis, PolC/β for one strand and DnaE/β for the other. Alternatively, the DnaE polymerase may serve an essential role in the replication process besides bulk replication. For example, DnaE may be required in initiation of replication, the processing of Okazaki fragments, or perhaps in the process of replication restart after replication fork encounter with damaged DNA. Interaction of DnaE with other members of the DNA synthetic machinery and its role in chromosome maintenance or replication remains an exciting topic for the future.

Comparison of Evolutionary Divergent Chromosomal Replicases—The Gram-positive and -negative cell lines split over one billion years ago. Nonetheless, genes encoding most of the protein subunits necessary for rapid and processive DNA synthesis have been conserved. However, the holA gene encoding the δ subunit has diverged significantly. The match observed in homology searches using E. coli δ as a query is so low that holA has not been identified in genome sequencing projects of other bacteria except for the rather closely related Gram-negative organism, H. influenzae (68).

FIG. 10. Interchange of S. pyogenes and E. coli replicase components. Interchange of S. pyogenes and E. coli replicase components. Mixtures of the polymerase, clamp, and clamp loader components of the S. pyogenes and E. coli replication systems were preincubated with primed M13mp18 ssDNA, and then a pulse of DNA synthesis was initiated. Reactions were rapidly quenched and then analyzed for RFII product formation in a native agarose gel as detailed under “Experimental Procedures.” Component mixtures are indicated above the autoradiogram of the gel. The positions of duplex product (RFII) and initial primed M13mp18 (ssDNA) are indicated to the right of the autoradiogram.
FIG. 11. The DnaE polymerase functions with β. Different amounts of DnaE polymerase were analyzed in a time course of primer extension on a SSB-coated M13mp18 ssDNA circle primed with a single DNA oligonucleotide. DNA products were analyzed in a native agarose gel. Reactions were performed in the presence of DNA oligonucleotide. Positions of initial primed M13mp18 (ssDNA) and of TMAU on PolC and DnaE in the presence of DNA. Positions of completed duplex (RFII) and initial point mutant in which the core polymerase to utilize a clamp loader that requires three different subunits to function and a circular β clamp that tethers the polymerase to DNA. Each case, the resulting replicase is remarkably rapid, ~700–800 nucleotides/s. Using the sequence of either the S. pyogenes δ or E. coli δ to search all the bacterial genomes, which have been completely sequenced, shows that all eubacteria thus far contain the holoA encoding δ, as well as genes encoding α, τ, δ', δ, and SSB. Therefore, on the basis of the information presented in this report on S. pyogenes and the previous studies in the E. coli system, it should now be possible to reconstitute the replicase of any eubacterial species from the genomic sequence alone.

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