The δ and δ* Subunits of the DNA Polymerase III Holoenzyme Are Essential for Initiation Complex Formation and Processive Elongation*

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δ and δ* are required for assembly of the processivity factor β2 onto primed DNA in the DNA polymerase III holoenzyme-catalyzed reaction. We developed protocols for generating highly purified preparations of δ and δ*. In holoenzyme reconstitution assays, δ* could not be replaced by δ, τ, or γ, even when either of the latter were present at a 10,000-fold molar excess. Likewise, δ could not be replaced by δ*, τ, or γ. Bacterial strains bearing chromosomal knockouts of either the holA (δ) or holB (δ*) genes were not viable, demonstrating that both δ and δ* are essential. Western blots of isolated initiation complexes demonstrated the presence of both δ and δ*. However, in the absence of ψ and single-stranded DNA-binding protein, a stable initiation complex lacking δδ* was isolated by gel filtration. Lack of δ-δ* decreased the rate of elongation about 3-fold, and the extent of processive replication was significantly decreased. Adding back δ-δ* but not ψ, δ, or δ* alone restored the diminished activity, indicating that in addition to being key components required for the β loading activity of the DnaX complex, δδ* is present in initiation complex and is required for processive elongation.

High processivities and fast elongation rates are hallmarks of the chromosomal replicates of eukaryotes and prokaryotes (1–3). The DNA polymerase III holoenzyme of Escherichia coli is the only enzyme capable of replicating its 4-megabase genome (4). The holoenzyme can synthesize DNA at a rate of ∼1 kilobase per s without dissociation from the template (5, 6). The process by which the DNA polymerase III holoenzyme catalyzes chromosome replication is complex; however, the fundamental mechanisms of action and the associated protein complexes appear to be evolutionarily conserved (3).

The E. coli DNA polymerase III holoenzyme, widely considered to be a prototypical replicative complex, is composed of 10 different subunits (α, ε, θ, β, τ, γ, δ, δ*, χ, and ψ). These subunits are organized into three types of functional complexes as follows: (i) a pol III1 DNA polymerase (pol III, αεθ), (ii) a processivity factor (β2 clamp), and (iii) an energy-dependent clamp loading apparatus (DnaX complex, τγψδδ*ψδ) (7–9). Both the τ and γ subunits are products of the dnaX gene; γ is a truncated version of τ arising from a –1 ribosomal frameshift (10–13). Both DnaX proteins associate with the auxiliary subunits δδ* and ψδ* to form a functional clamp loader. Chemical crosslinking was recently used to demonstrate that δ* and ψ contact γ and not τ within the native holoenzyme (14). The auxiliary and catalytic subunits function together to confer upon the holoenzyme special properties that distinguish it from simpler polymerases not devoted to chromosome replication. In the presence of auxiliary subunits, ATP is ineffective in inducing the formation of stable complex dramatically decreasing processivity (15). pol III (αεθ) activity is inhibited by high salt concentrations and the E. coli single-stranded DNA-binding protein (SSB) in the absence, but not the presence, of auxiliary subunits (5, 16). Thus, the auxiliary subunits appear to assume these inhibitory effects in simpler polymerase forms.

The δ and δ* subunits are key components for the assembly and function of the DnaX complex (τγδδ*ψδ, γδδ*ψδ, γτγδδ*ψδ, or τγδδ*ψδ) and are required for the assembly of the processivity factor β2 onto primed template (17, 18). Previous studies have shown that δ and δ* are distinct proteins encoded by different genes (holA (δ) and holB (δ*)) with molecular masses of 38.7 and 36.9 kDa, respectively. These two genes were recently identified, cloned, and overexpressed (18–21). The δ subunit directly interacts with β within the DnaX complex (8) and is required not only for the loading of the β2 processivity factor around DNA but also for its dissociation from the template (22). δ* is a key assembly factor that binds both DnaX and δ, bridging them together (23, 24). Both δ and δ* stimulate the ATPase activity of DnaX proteins, τ and γ (17, 18). Despite its unique features, δ* shares 28% sequence identity with the γ subunit (21, 25). This striking example of redundancy between subunits within the DnaX complex is echoed in other homo-

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§ The abbreviations used are: pol III, DNA polymerase III (αεθ); DnaX complex, a functional subassembly containing either or both DnaX proteins (τ and/or γ) with associated δ, δ*, χ, and ψ; DnaX protein, either of two alternative products of the dnaX gene (τ or γ); IC, initiation complex, a stable complex between the DNA polymerase III holoenzyme and primed DNA that is formed upon ATP hydrolysis; SSB, E. coli single-stranded DNA-binding protein; dADPNP, deoxyadenylylimidodiphosphate; r-complex, τγδδ*ψδ; γ-complex, γδδ*ψδ; BSA, bovine serum albumin; n-IC, an IC that was formed using the native holoenzyme and a 30-mer/M13<sub>mon</sub> primer-template; r-IC, an IC that was formed using pol III, β2, the r-complex, and a 30-mer/M13<sub>mon</sub> primer-template; γ-IC, an IC that was formed using pol III, β2, the γ-complex and a 30-mer/M13<sub>mon</sub> primer-template; IC–δδ*ψδ, an auxiliary subunit-deficient IC that was formed using pol III, β2, τ, δδ*, then gel filtered to remove δδ*; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; kb, kilobase pair; bp, base pair.
logues of the clamp-loader family including T4 phage gp44/62 proteins (26) and human replication factor-C (27). The crystal structure of the E. coli δ' recently has been solved and was used to provide a model for the structure of γ (25).

As described in this report, we developed purification procedures that yield large quantities of homogeneous δ and δ'. We have used these δ and δ' preparations in studies that further define their roles in replication catalyzed by the DNA polymerase III holoenzyme. The requirements for δ or δ' in DNA replication were investigated in vitro by use of a reconstitution activity assay employing homogenous subunits, and in vivo by use of bacterial strains carrying chromosomal knockout versions of either holA or holB. We also developed a method to isolate an IC lacking both δ and δ'. This allowed us to explore the roles of δ' subsequent to formation of the IC. Evaluation of the rate and extent of DNA chain elongation revealed that δ and δ' are involved in elongation.

**EXPERIMENTAL PROCEDURES**

**E. coli Strains and Growth—**Recombinant δ (19) and δ' (21) were overexpressed in E. coli strain HB101 bearing pJRl05 or pMAF205, respectively. (28) E. coli was grown on a 250-liter fermentor (New Brunswick Scientific) in F media and ampicillin at 37 °C as described (28). Glucose (1% (v/v)) and ampicillin (70 μg/ml) were added at the beginning of the fermentation and at the point of induction. Cells were grown to an optical density of 1.0 (600 nm), and then recombining protein expression was induced by addition of isopropyl-β-D-thiogalactoside (1 mM final concentration). Three hours after induction, cells were harvested as described (28).

**Buffers—**Tris/sucrose buffer is 50 mM Tris (pH 7.5) and 10% sucrose. Buffer A is 50 mM Tris (pH 7.4), 20% glycerol (v/v), 1 mM EDTA, and 5 mM DTT. Buffer B is 20 mM KPO4 (pH 7.4), 5% glycerol, 0.1 mM EDTA, and 5 mM DTT. Buffer C is 20 mM KPO4 (pH 7.4), 5% glycerol, 0.1 mM EDTA, 5 mM DTT, and 400 mM NaCl. Buffer D is 10 mM KPO4 (pH 7.4), 10% glycerol, and 1 mM DTT. Buffer E is 50 mM Tris (pH 7.0), 5% glycerol, 1 mM EDTA, and 5 mM DTT. Buffer F is 50 mM Hepes (pH 7.4), 10% glycerol, 10 mM MgCl2, 100 mM NaCl, and 10% glycerol. HBs buffer is 10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% (v/v) P-20 detergent (BIACore Inc., Piscataway, NJ). HKGM buffer is 50 mM Hepes (pH 7.4), 100 mM potassium glutamate, 10 mM Mg(OAc)2, and 0.005% (w/v) P-20 detergent.

**Chromatographic Supports—**Q-Sepharose, SP-Sepharose, Sephacryl S-100, and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Hypatite C (hydroxylapatite, lot number 6644) was from Hypatite C (hydroxylapatite). Trilink Biotech Inc. (San Diego, CA). 7S-100, and protein G-Sepharose were obtained from Amersham Pharmacia Biotech. Hypatite C (hydroxylapatite, lot number 6644) was from Hypatite C (hydroxylapatite).

**Purification of δ Subunit**

**Cell Lysate Preparation and Ammonium Sulfate Precipitation—**All procedures were performed at 4 °C unless otherwise stated. E. coli strain HB101, human replication factor-C (DMSO) (28), and holB were purified at a level of 2–4% of total protein (19). 750 g of cells were resuspended in 3.75 liters of Tris/sucrose buffer. Lysozyme (final concentration, 0.6 mg/ml) was added, and cells were incubated for 1 h on ice then heat-treated at 37 °C for 5 min. The lysate was centrifuged at 22,000 × g for 1 h to remove cell debris. Supernatants were pooled to yield fraction I (28). δ in fraction I was precipitated with ammonium sulfate (0.242 g per ml of fraction I, 40% saturation) and centrifuged at 22,000 × g for 30 min. Pellets were washed with resuspension in a Dounce homogenizer with Buffer A (0.2 g of ammonium sulfate was added to each milliliter, 35% saturation) and centrifuged as before. The pellets were flash-frozen in liquid nitrogen and stored frozen at −80 °C as fraction II (Table I).

**Q-Sepharose Chromatography—**Fraction II was resuspended in 300 mM of Buffer A and dialyzed against Buffer A + 50 mM NaCl (pH 7.0). Unless otherwise stated, all dialysis procedures described in this report employed a 45-mm membrane with a molecular mass cut of 3,500 Da (Spectrum Laboratories, Inc., Rancho Dominguez, CA). After dialysis, absorbance was checked three times, 5 liters each, every 3 h. The dialyzed δ fraction II sample was diluted to a conductivity equivalent to Buffer A + 50 mM NaCl by adding Buffer A and then applied to a Q-Sepharose FF column (42.4 cm2 × 25 cm). The δ subunit requires DTT for maintenance of activity and tends to precipitate in low salt (<70 mM), especially during dialysis. Thus, low salt buffers were avoided except for short periods, and 5 mM DTT was included in all buffers. The column was washed with 8 liters of Buffer A + 100 mM NaCl, and proteins were eluted with a 0.1-liter/NaCl gradient (130–200 mM) in Buffer A at a rate of 150 ml/h. δ was eluted at a conductivity equivalent to Buffer A + 150 mM NaCl. Fractions were pooled at one-half peak height by activity as fraction III (1.9 liters, Table I).

**SP-Sepharose Ion Exchange Chromatography—**δ in fraction III was precipitated with ammonium sulfate (60% saturation) and centrifuged at 23,000 × g for 1 h. The fraction III pellet was resuspended in 200 ml of Buffer A and dialyzed against Buffer A + 30 mM NaCl. The dialysate was clarified by centrifugation (23,000 × g for 30 min) and applied to a 350-ml SP-Sepharose HR column equilibrated with Buffer A + 30 mM NaCl at a flow rate of 100 mM NaCl. The column was washed with 7 liters of Buffer A + 30 mM NaCl, and proteins were eluted with a 3.8-litre/NaCl gradient (30–200 mM) in Buffer A at a flow rate of 80 ml/h, δ was eluted at a conductivity equivalent to Buffer A + 100 mM NaCl. Fractions were pooled at one-half peak height by activity, as fraction IV (990 ml, Table I).

**Sephacryl S-100 Gel Filtration Chromatography—**δ in fraction IV was precipitated with ammonium sulfate (55% saturation) and centrifuged at 23,000 × g for 1 h. The pellet was resuspended in 33 ml of Buffer C, centrifuged at 23,000 × g for 30 min, and then applied to a Sephacryl S-100 column (5.7 cm2 × 110 cm) equilibrated with Buffer C. The δ subunit (eluted at 3.1 ml/fraction) with Buffer C at a flow rate of 36 ml/h. Activity peak fractions were combined to yield fraction V (65 ml, Table I). Purified δ (Fig. 1A) was aliquoted, immediately frozen in liquid nitrogen, and stored at −80 °C.

**Purification of the δ' Subunit**

**Cell Lysate Preparation and Ammonium Sulfate Precipitation—**E. coli strain HB101 containing pMAF205 (DMSO number 886) was used.
TABLE II

| Fraction of δ’ | Total protein (mg) | Activity (units/mg) |
|---------------|-------------------|---------------------|
| I. Cell lysate | 16,000            | 2.3 × 10^7         |
| II. Ammonium sulfate | 3,000            | 7.7 × 10^10        |
| III. Q-Sepharose | 490              | 4.4 × 10^10        |
| IV. Hydroxylapatite | 390             | 4.9 × 10^10        |
| V. Q-Sepharose | 170               | 3.9 × 10^10        |
| VI. Sephacryl S-100 | 150*             | 3.6 × 10^10        |

* The amount of δ’ from fraction VI was 104 mg as determined using the extinction coefficient of purified δ’ (ε₂₆₀ = 59,726) (9).

The sedimentation coefficient calculated when protein was determined under Footnote a was 2.53 × 10^6 units/mg.

this strain produces δ’ at levels corresponding to ~2% of total protein. Cells were suspended to 20% (v/v) in Tris/sucrose buffer. Cell lysate preparation (fraction I) and ammonium sulfate precipitation (fraction II) were performed as described for the δ subunit.

Q-Sepharose Chromatography—δ’ fraction II was resuspended in 300 ml of Buffer E + 20 mM NaCl and dialyzed against this buffer. Care should be taken at this step to limit dialysis time to 6 h or less to avoid precipitation of δ’ activity. The sample was then diluted to a conductivity equivalent to Buffer E + 20 mM NaCl by adding Buffer E and applied to a Q-Sepharose FF column (42.4 cm² × 25 cm). The column was washed with 7.2 liters of Buffer E + 20 mM NaCl, and proteins were eluted with an 8-liter NaCl gradient (20–200 mM) in Buffer E at a flow rate of 180 ml/h. δ’ eluted at a conductivity equivalent to Buffer E + 110 mM NaCl. Fractions were pooled at one-half peak height by activity as fraction III (Table II).

Hydroxylapatite Chromatography—δ’ fraction III was precipitated with ammonium sulfate (55% saturation) and centrifuged at 23,000 × g for 1 h. The fraction III pellet was resuspended in 300 ml of Buffer D and dialyzed against this buffer (2 changes every 2 h, 5 liters each). The dialysate was clarified by centrifugation (23,000 × g, 30 min) and applied to a 350-ml hydroxylapatite column equilibrated with Buffer D at a flow rate of 90 ml/h. The column was washed with 1.5 liters of Buffer D, and proteins were eluted with a 3-liter KPO₄ gradient (10–150 mM) at a flow rate of 90 ml/h. Fractions were eluted at a conductivity equivalent to Buffer D + 60 mM KPO₄ and pooled at one-half peak height by activity, as fraction IV (630 ml). The contaminating nucleic activity of the pooled fraction IV of δ’ was 33 units per 20 µg as determined by a nucleic acid assay employing a double-stranded DNA substrate. No single-stranded DNA-specific nucleic activity was detected in δ’ fraction IV.

Q-Sepharose Chromatography II—An additional anion exchange column was used to remove the contaminating nucleic acids that co-eluted with δ’ in the hydroxylapatite column chromatographic step. δ’ in fraction IV was precipitated with ammonium sulfate (55% saturation) and centrifuged before the fraction IV pellet was resuspended in 70 ml of Buffer E + 20 mM NaCl and dialyzed against this buffer (2 changes every 2 h, 5 liters each). This fraction was then applied to a Q-Sepharose FF column (350 ml) pre-equilibrated with Buffer E + 20 mM NaCl. The column was washed stepwise with 1.8 liters of Buffer E + 20 mM NaCl followed by 2-liter Buffer E + 90 mM NaCl. Proteins were eluted at 40 ml/h with a 2-liter NaCl gradient (100–200 mM) in Buffer E. δ’ eluted at a conductivity equivalent to Buffer E + 140 mM NaCl; the contaminating nucleic activity was successfully removed by this procedure. The eluted δ’ fractions were pooled at one-half peak height by activity as fraction V (425 ml, Table II). Contaminant nucleic activity (double-stranded DNA) in the pooled fraction V of δ’ was <4 units per 20 µg.

Sephacryl S-100 Gel Filtration Chromatography—δ’ in fraction V was precipitated with ammonium sulfate (55% saturation) and centrifuged as before. The fraction V pellet was resuspended in 25 ml of Buffer B, clarified by centrifugation (23,000 × g, 30 min), and applied to a Sephacryl S-100 column (5.7 cm² × 90 cm). The δ’ subunit was eluted (7 ml each) with 1.1 liters of Buffer B at a flow rate of 30 ml/h. Activity peak fractions were combined to yield fraction VI (70 ml, Table II). Purification δ’ (Fig. 1B) aliquots were immediately frozen in liquid nitrogen and stored at −80°C.

Construction of holA(OC) and holB(OC) Strains—Strains carrying holA and holB ocher alleles were constructed using the gene replacement system described by Link et al. (37). Genomic DNA from strain KA796 (ara, thi, ΔprosA) (38) was isolated using the Easy-DNA kit (Invitrogen, Carlsbad, CA). The holA and holB genes were polymerase chain reaction-amplified (25 cycles each of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C) using Pfu polymerase and primer sets A-1 and A-2 for holA or B-1 and B-2 for holB, respectively. The primers were designed to enable amplification of the complete holA or holB gene plus flanking regions of 500–600 base pairs on either side. In addition, the primers were designed to create BglII or SfiI sites for the holA gene and BglII sites for the holB gene, respectively. The resulting polymerase chain reaction products were extended twice with phenol:chloroform:isoamyl alcohol (25:24:1) and digested with BglII or SfiI. The digested fragments were inserted by ligation into the BamHI site of gene replacement vector pK03 (37). This plasmid, which provides chloramphenicol resistance at temperature(s) and allows con- stitution of the gene clone: chloramphenicol at 30 °C, was introduced into E. coli by electroporation, and chloramphenicol-resistant transfectants were isolated on LB-chloramphenicol plates at 43 °C. Due to the inability of pK03 to replicate at 43 °C, the only transfectants obtained at this temperature are recombinants in which the plasmid has integrated, by homologous recombination, into the chromosome using the homology between the plasmid insert and the corresponding chromosomal sequence (37). The result is a partial diploid strain carrying two copies of the gene of interest (one wild-type and one mutant) separated by the plasmid sequence. Subsequent growth of the transfectants at 30 °C allows for plasmid excision by reversal of the process. Either the wild-type or the mutant copy may be excised, leaving behind the other copy in the chromosome. Several individual transfectants for each construct were picked, inoculated into 5 ml of LB-chloramphenicol, and grown to saturation at 30 °C. The cultures were then subjected to two sequential cycles of 100-fold dilution and growth in LB-chloramphenicol at 30 °C. The final cultures were inoculated onto LB-chloramphenicol plates and grown at 30 °C. Single colonies were resuspended in LB broth, dilutions spotted on LB plates, and then grown at 30 °C or 43 °C. This revealed temperature-sensitive segregants that carry the wild-type holA or holB gene plus the ocher mutant version of the chromosome. Plasmid from the temperature-sensitive isolates was sequenced, sequenced, and confirmed to carry the wild-type gene. The temperature-sensitive segregants were converted to their recA56 derivative by P1 transduction using linkage with src::Tn10, yielding recA strains NR12807 (holA(Oc)pOAc) and NR12846 (holB(Oc)pOAc). The control strain is NR11284 (KA796, recA56, src::Tn10).

Homology-directed repair (HDR) plasmid variants were prepared: n-IC, t-IC, γ-IC, and IC(δ’ψφ) (39). The first three variants were made because we wanted to compare a reconstituted IC that roughly corresponds to IC formed using native holoenzyme isolated from wild-type cells (which contains both γ and χ) with variants containing only one of the two DNA proteins. These IC preparations were generated by using either the native DNA polymerase III holoenzyme or a reconstructed holoenzyme along with either of two DnaX complexes (the t-complex, γδφψφψ, or the γ-complex, γδφψψψψ). A 30-mer/ M13α, primer-template. Complexes formed using the native holoenzyme are designated n-IC; those generated using reconstituted holoenzyme along with the t- and γ-complexes are denoted t-IC and γ-IC, respectively. To generate n-IC, purified native holoenzyme (0.25 nmol) was incubated with 30-mer/M13α::Tn5 (0.025 nmol as circle) at 30 °C for 5 min in 50 mM Hepes (pH 7.4), 0.5 mM MgATP, 10 mM MgOAc, 5 mM DTT, 100 mM NaCl, and 10% glycerol in 150 µl total volume. The t-IC and γ-IC complexes were produced by incubating a 10-fold molar excess of reconstituted holoenzyme generated from pol III (1 nmol), β* (1 nmol), and the relevant DnaX complex (0.6 nmol), with 30-mer/ M13α::Tn5 (0.05 nmol as circle) under the same conditions, except that the temperature was 30 °C and 730 µmol of ATP at a concentration of 0.8 µmol/mg for the reaction. To generate (IC-δ’ψφ) was first generated in the presence of δ’ and δ’; the latter two subunits were then removed by dissociation during gel filtration. To construct IC(δ’ψφ), reaction mixtures (150 µl) containing pol III (1 nmol), β* (1 nmol), τα (0.5 nmol), δ’ (0.5 nmol), and δ’ (0.5 mol) were incubated
at 30 °C for 5 min. 150 μl of 30-mer/M13(ori) (0.05 nmol as circle) in 50 mM Hepes (pH 7.4), 0.5 mM ATP, 10 mM Mg(OAc)2, 5 mM DTT, 100 mM NaCl, 10% glycerol were then added; the mixtures were incubated for an additional 3 min at 30 °C then diluted to a final volume of 600 μl via addition of Buffer F. An appropriate control for use in experiments employing [\(\delta^32P\)dGTP] was generated in sister reactions performed exactly as above except for the inclusion of χ(0.5 nmol). Each type of IC was gel-filtered through Bio-Gel A-5m columns equilibrated with Buffer F immediately subsequent to generation. The IC variants were filtered through columns (1.0 cm × 24 cm) at a flow rate of 0.7 column volume/h. IC-[\(\delta^32P\)dGTP] and the control IC were filtered through larger columns (1.1 cm × 48 cm), at the slower flow rate of 0.1 column volume/h, which facilitated dissociation of the δ and χ′ subunits from IC-[\(\delta^32P\)dGTP] to generate IC-[\(\delta^32P\)dGTP]. All four types of ICs were quantified by measuring DNA synthesis after the addition of [\(\delta^3H\)]dNTPs as described previously (35).

Nuclease Detection Assay—A sensitive nuclease assay was developed to detect and quantify contaminating nucleases in δ′ fractions. The nuclease substrate was a [\(\delta^3H\)]dTMP incorporated replicative form of M13(ori) DNA that was precipitated using standard holoenzyme activity assay. Double-stranded DNA (700 nmol) was incubated with 20 μg of δ′ fraction in 20 mM KPO4 (pH 7.4), 25 mM NaCl, 1 mM MgCl2, 15% glycerol, 1 mM DTT (final volume, 100 μl) at 30 °C for 30 min. Reactions were quenched by addition of trichloroacetic acid, and the radioactivity in precipitates was quantified as described below. To detect single strand-specific nucleases, the replicative form of double-stranded [\(\delta^3H\)]dGTP DNA was melted by heating at 90 °C for 10 min. DNA samples were then promptly chilled on ice for 5 min prior to assay. To detect exonuclease activity, double-stranded [\(\delta^3H\)]M13(ori) DNA was treated by DNase1 to generate a gapped DNA (39), and the nuclease activity was determined from a primed M13(ori) template as described (24). In these assays, the amount of total nucleotide incorporated into acid-insoluble DNA was determined. Reactions were carried out at 30 °C for 5 min and quenched with trichloroacetic acid. One unit is defined as the amount of enzyme required to catalyze the incorporation of 1 pmol of total deoxyribonucleotide per minute at 30 °C. Digestion assays using 30-mer/M13(ori) primer-template were performed under the standard reconstitution assay conditions except that 500 pmol of 30-mer annealed M13(ori) DNA was substituted as a primed template and that SSB, primer, and dNTPs were omitted.

IC Activity Assay—Unless otherwise indicated, the replication activity of ICs was assayed by addition of 48 μM each of dATP, dCTP, and dGTP, as well as 18 μM [\(\delta^3H\)]dTTP (specific activity of 540 cpn/mmol dTTP) in 50 mM Hepes (pH 7.4), 10 mM Mg(OAc)2, 0.1 mM ATP, 5 mM DTT, 0.1 mM potassium glutamate, and incubated for specified times at 30 °C. Reactions were quenched with trichloroacetic acid, and acid-precipitable radioactivity was measured. Prior to performing IC activity assays in which [\(\delta^3H\)]dDNPNP was used in lieu of dATP, the residual ATP in IC preparations was first removed by Bio-Gel A-5m gel filtration (1.1 cm2 × 48 cm). The assay conditions were as described above, except that 48 μM dADPNP replaced dATP, and ATP was not included.

Analysis of DNA Elongation Rate—The DNA synthesis rates of isolated IC and IC-[\(\delta^32P\)dGTP] were determined by a modification of Fay et al. (16) and Kim and McHenry (39). A reaction mix containing isolated IC in 50 mM Hepes (pH 7.5), 10% (v/v) glycerol, 0.1 mM potassium glutamate, 10 mM DTT, 10 mM Mg(OAc)2, 200 μM/ml BSA, 0.02% (v/v) Tween 20, and 250 μM ATP was incubated at room temperature for 3 min, and the DNA synthesis reaction was initiated by the addition of 48 μM each of dCTP, dATP, and dTTP as well as 18 μM of [\(\delta^32P\)dGTP (specific activity = 3,000 cpn/mmol, as dNTPs) at 30 °C. Reactions were quenched at the indicated times by transferring an aliquot of a given sample to a sterile Microcentrifuge tube containing 0.5 volume of 7.5 M ammonium acetate that had been pre-chilled in a dry ice/ethanol bath. DNA samples were precipitated on ice for 1 h and centrifuged (15,000 × g, 30 min, 4°C). Pellets were resuspended in 30 μl of 50 mM potassium phosphate (pH 7.9), 20 mM Tris acetate, 10 mM Mg(OAc)2, and 1 mM DTT. The DNA was digested with 6 μl of Drai (20,000 units/ml) in a total volume of 40 μl at 37 °C for 5 h, loaded on an 8% native polyacrylamide gel (1.5 × 25 × 15 cm), and electrophoresed at 150 V overnight. A Molecular Dynamics PhosphorImager was used to quantify the α–32P-labeled DNA.

RESULTS

δ and δ′ Are Essential in DNA Replication Both in Vitro and in Vivo

A Molar Excess of DnaX (τ/γ) Cannot Overcome the Requirement for Either δ or δ′—As described under “Experimental Procedures,” we developed purification procedures for obtaining highly purified recombinant δ and δ′ in quantities sufficient for investigating the roles of these auxiliary subunits in DNA replication. In representative experiments, these protocols yielded 176 and 130 mg of homogenous δ and δ′, respectively (Fig. 1, Tables I and II). The purities of the preparations were examined via Western blots employing subunit-specific monoclonal antibodies (data not shown). No cross-contamination of the δ preparations with δ′ or vice versa was detected. Furthermore, no contamination of preparations of either the δ or δ′ subunits with the τ, χ′, or ψ subunit of holoenzyme was observed. These preparations were utilized in modified reconstitution activity assays to assess whether δ and/or δ′ are necessary for holoenzyme-catalyzed DNA replication. These tests were used to determine whether holoenzyme activity could be reconstituted in the absence of either δ or δ′ by adding molar excesses of other constituent subunits. Holoenzyme activity can be reconstituted even with trace amounts of the protein (2–5 fmol), and thus, this type of assay can also serve as a rigorous test to detect contaminating holoenzyme or constituent subunits in preparations of a given isolated subunit. 2
cause previous studies suggested that $\delta$ with a molar excess of DnaX is capable of loading $\beta$ clamp onto DNA in the absence of $\delta'$, we first examined whether high levels of $\gamma'\gamma$ can replace $\delta'$. We found that, however, addition of up to a 10,000-fold molar excess of $\tau$ (Fig. 2A) or $\gamma$ (Fig. 2B) did not result in the reconstruction of holoenzyme activity in the absence of $\delta'$. Similarly, large molar excesses of DnaX protein failed to support reconstruction of holoenzyme activity in the absence of $\delta$ (Fig. 2, A and B). The requirement for neither $\delta$ nor $\delta'$ was alleviated by prolonging the incubation time (from 20 s to 15 min). Thus, both $\delta$ and $\delta'$ were absolutely required for the reconstruction of DNA polymerase III holoenzyme activity (Fig. 2).

A Molar Excess of $\delta$ Could Not Overcome the Requirement for $\delta'$—Having demonstrated that $\delta'$ is not replaced by an excess of DnaX ($\gamma'\gamma$), we examined whether an excess of $\delta$ could replace the requirement for $\delta'$. Increasing concentrations of $\delta$ were added in the presence or absence of $\delta'$ (Fig. 3A). A 30,000-fold molar excess of $\delta$ (72 $\mu$m) could not substitute for $\delta'$. Similarly, a large molar excess of $\delta'$ could not replace the requirement for $\delta$ (Fig. 3B).

The holA and holB Genes That Encode $\delta$ and $\delta'$ Are Essential Genes—To confirm the biochemical requirements for both $\delta$ and $\delta'$ in vivo, we constructed bacterial strains carrying chromosomal knockouts of either holA or holB. Tandem double-ochre codons were introduced to the respective genes of the bacterial chromosome for this purpose. To compensate for the holA or holB deficiency, these strains also carry the corresponding wild-type holA or holB gene on a plasmid (see “Experimental Procedures”). Plasmid pKO3 (37) contains a temperature-sensitive pSC101 origin permitting replication at 30 °C but not at 42 °C. Thus, a test of these constructs to form colonies at 42 °C, at which the plasmid cannot duplicate and segregate into newly made daughter cells, can serve as a direct test for the essentiality of the holA and holB genes. The strains used were also recA to prevent recombination between the plasmid and the chromosome. At 30 °C, each of the three strains used, the parental control, the holA(oc)/pOA strain, and the holB(oc)/pOB, were able to grow normally, but at 42 °C only the control strain was capable of forming colonies (Fig. 4). These data demonstrate that the presence of the wild-type holA or holB gene is required for the cell growth, and thus, both holA and holB are essential.

$\delta$ and $\delta'$ Are Part of the IC

ICs Formed with Native Versus Reconstituted Holoenzyme—To extend further our knowledge regarding the functional roles of $\delta$ and $\delta'$ in DNA replication, we next sought to determine whether these subunits are contained in the IC. ICs were prepared by using either reconstituted DNA polymerase III holoenzyme or the native holoenzyme. SSB is known to bind the DnaX complex ($\gamma\delta\delta'\chi\phi$) (30, 40) and was found to interfere with isolation of initiation complexes free of exogenous DnaX complexes because of $\gamma$-SSB interaction (data not shown). To prevent association of DnaX complex components with SSB-coated DNA, the requirement for SSB and DnaG primase was bypassed by annealing a DNA 30-mer primer to a M13 Gori template. The replication of 30-mer/M13 Gori primer-template by holoenzyme was comparable to that of primase-primed SSB-coated M13 Gori DNA. Furthermore, ICs were gel-filtered in a buffer containing an elevated level of salt (100 mM NaCl) to

d and $\delta'$ Facilitate the Efficient DNA Chain Elongation

Fig. 2. Excess of $\tau$ or $\gamma$ cannot overcome the requirement for either $\delta'$ or $\delta$ for the replication activity of holoenzyme. Holoenzyme reconstitution activity assays were performed in the absence of either $\delta'$ or $\delta$. Each assay contained the indicated amount of $\tau$ or $\gamma$, pol III (500 fmol), $\beta_1$ (500 fmol), $\chi\phi$ (500 fmol), and SSB-coated M13 ori (540 pmol, as nucleotide) in the presence or absence of $\delta$ (100 fmol) and/or $\delta'$ (100 fmol). All remaining assay components were mixed and incubated at 30 °C for 5 min as described under "Experimental Procedures." Varying amounts of the $\tau$ subunit (A) or the $\gamma$ subunit (B) were added to each assay in absence of either $\delta'$ (closed cross) (the symbol is obscured in the graph because of overlap with other symbols), $\delta$ (closed triangle), or $\beta\beta'$ (closed circle), or in the presence of both $\delta$ and $\delta'$ (closed square).

Fig. 3. Excess $\delta$ could not replace $\delta'$. Holoenzyme reconstitution activity assays were performed in the absence of either $\delta$ or $\delta'$. A, the indicated amounts of $\delta$ were added to assay mixtures containing 500 fmol of each $\alpha$, $\epsilon$, $\theta$, $\gamma$, $\chi$, $\phi$, $\delta$, and $\beta$ in the absence (closed inverted triangle) or in the presence (closed circle) of $\delta'$ (100 fmol). B, the indicated amounts of $\delta'$ were added to assay mixtures containing 500 fmol of each $\alpha$, $\epsilon$, $\theta$, $\gamma$, $\chi$, $\phi$, and $\beta$ in the absence (closed triangle) or in the presence (closed circle) of $\delta$ (100 fmol).
mitigate non-specific interactions between protein and DNA
(Fig. 5, A–C, lane 17). IC made by using either the native
holoenzyme (n-IC) or by using holoenzyme reconstituted by
τ-complex (τ-IC) contained both δ and δ' subunits as well as all
other holoenzyme components as visible in Coomasie-stained
SDS-polyacrylamide gel3 (Fig. 5, A and C). τ-IC and n-IC had
comparable replication activities (Fig. 5, D and F). The pres-
ence of both δ and δ' in both ICs was confirmed by Western blot
analyses using subunit-specific monoclonal antibodies (Fig. 5,
D–F). n-IC contained the γ-, δ-, δ', χ-, and θ- subunits, suggest-
ing that each of these auxiliary subunits are present in natu-
urally occurring IC.

Yields of activity recovery upon gel filtration for n-IC and
τ-IC, were 39 and 41%; and protein recoveries were 71 and 83%
respectively, indicating the comparability between the two ICs
in the absence of SSβ at elevated levels of salt. However,
whereas n-IC contains a mixed γ/τ DnaX complex, and τ-IC was
shown to contain the τ-complex, IC generating using the γ-com-
plex (γ-IC) differed in that the final product did not contain any
of the components of the γ-complex (Fig. 5B, lane 17) (41). Fur-
thermore, the apparent ratio of β2 to pol III of the isolated
γ-IC was −7-fold higher, and the replication activity of this
complex was 10–15-fold lower than that of n-IC or τ-IC (Fig.
5E), suggesting that γ-IC is different both physically and
functionally.

In control experiments, complexes were also prepared and
gel-filtered in the absence of ATP or DnaX complex (Fig. 5,
G–I). In the absence of ATP, no detectable enzyme-DNA complex
survived gel filtration (Fig. 5G). When a 10-fold molar excess of pol III plus β (1.66 μM as pol III/β) to the primer-
template (0.16 μM as circle) was incubated under the same conditions used to generate ICs, except that no DnaX complex
was included, the pol III/β complex did not co-elute together
with the DNA (Fig. 5H). The lack of non-specific adherence of
the δδ′ and the other subunits of the τ-complex (τ̂δδ′ ψ) to DNA
was confirmed by incubating a 10-fold molar excess of DnaX complex with 30-mer/M13 DNA prior to the gel filtration
procedure (Fig. 5J). None of these controls showed activity upon
addition of dNTPs (Fig. 5, G–I).

3 The dye binding ratio corrected only for differences in molecular
weight was −α2 (τ̂δδ′ ψ) / β1 ρ0 δδ′ ψα2 (τ̂δδ′ ψ) / ρ0 δδ′ ψ, for τ-IC, and
−α1, τ̂δδ′ ψ / β1 ρ0 δδ′ ψα1, τ̂δδ′ ψ / ρ0 δδ′ ψ, for n-IC. Under the experimental
conditions used, we observed that a small fraction of β subunit was
often dissociated during the gel filtration. Contaminants in native ho-
loenzyme preparations co-migrated with the ε subunit and were not
resolvable on 4–20% gradient SDS-PAGE. The ratios shown here were
estimated by densitometry of Coomasie-stained SDS-polyacrylamide
gels.

δδ′ Participate in Elongation

IC Lacking δδ′ Has Decreased Activity—It has been demon-
strated that δδ′ is required for the assembly of processivity
factor β onto primed DNA (8, 17). However, roles for δδ′ in
holoenzyme function subsequent to β3 assembly have not been
reported previously. By having demonstrated that δ and δ' are
parts of the IC, we next sought to determine whether δδ′ might
participate in elongation. If δδ′ have a function after loading β
onto DNA, then the IC lacking δδ′, in which pol III/β3 were
properly assembled on a primed template, may function differ-
tently in elongation than does IC. To explore the role for δδ′ in
elongation, we developed a method to physically isolate an IC
lacking both δ and δ’. Because ψ increases the affinity of δδ′ for
DnaX (τ/γ) by 10–20-fold (24, 42), yet is nonessential in
single-stranded M13 DNA replication system (24, 43), we omit-
ψ in our δδ′ subunit-deficient IC preparation (designated
IC-[δδ′ ψ]). To favor the dissociation of δδ′ from IC, the samples
were also diluted and gel-filtered with a slow flow rate to give
δδ′ time to dissociate. Size exclusion gel filtration analysis
indicated that δδ′ was successfully removed under these condi-
tions (Fig. 6A). The void volume fraction contained neither δ
nor δ’, as detected by the Coomasie-stained SDS-PAGE gels
(Fig. 6A, fractions 12 and 13). Absence of both δ and δ’ were
further confirmed by Western blot analyses using anti-δ or
anti-δ’ monoclonal antibodies (data not shown). In parallel, IC
was formed with the reconstituted holoenzyme (pol
IIIβ3δδ′ ψ) under the same conditions (Fig. 6B). This control
IC (τ-IC) contained δδ′ as well as all other holoenzyme subunits
(Fig. 6B). From the densitometric scan of the gel with known
amounts of standard subunits, we found that both the control
IC and IC-[δδ′ ψ] contained comparable amounts of a (Table
III), e, β, and τ, respectively. These results indicate that lack
of these auxiliary subunits does not significantly affect the tight
complex assembly of pol III/β3 on primed template.

Prior to gel filtration, the replication activity of IC in the
absence of ψ was highly active and indistinguishable from
that of the control IC. However, after gel filtration, the yield
of activity recovery was decreased about 8-fold as compared with
the control IC. Yet protein recoveries in both void volume
fraction as well as total column fractions were similar (71
versus 65%, respectively). Moreover, the replication activity per
unit volume was lower more than 9-fold in IC-[δδ′ ψ] (Table
III). Consistent with these observations, the sum of activity per
polymerase a was decreased by 10-fold (Table III) as compared
with the control IC. These results indicate that in the presence
of the τ subunit, pol III-β is relatively stable on DNA regardless
of whether δδ′ ψ is present; however replication activity is not
appreciable without δδ′ ψ.

δδ′ ψ Facilitates the Rate of DNA Synthesis—We determined
the rate of DNA synthesis to evaluate whether the diminished
activity of IC-{δδ′χψ} is due to a defect on DNA chain elongation. The availability of physically isolated IC and IC-{δδ′χψ} permitted us to investigate the elongation rate by using restriction digestion analysis (16, 39). DNA synthesis was initiated by the addition of [α-32P]dNTPs at 30 °C (Fig. 7). The DNA fragments generated from Drai digestion are a (283 bp), b (365 bp), c (290 bp), d (950 bp), e (2585 bp), and f (4150 bp) in the order produced started from 3'-OH end of the annealed 30-mer primer on M13Gori DNA (Fig. 7C). Fragments b and d appeared at 20 s and at 3 min, respectively, for IC-{δδ′χψ} (Fig. 7A); and
Fig. 6. Isolation of the IC-[δβ' χφ]. A, IC-[δβ' χφ] was prepared with 10-fold excess of pol IIIββδδ72 and the ability to synthesize DNA upon addition of [3H]dNTP was the enzyme preparation (pol IIIββδδ72) and the ability to synthesize DNA upon addition of [3H]dNTP was the enzyme preparation (pol IIIββδδ72). Insets, shown are the Coomassie Blue-stained 4–20% gradient SDS-polyacrylamide gel of the activity peak fraction. 150 μl of each fraction from Bio-Gel-A5m column chromatography as described under “Experimental Procedures.” Column chromatography profiles of activity (closed circle) and protein (open square) are shown. 10 μl of each fraction from Bio-Gel-A5m column was assayed in a total volume of 25 μl in 50 mM Hepes (pH 7.4), 10 mM Mg(OAc)2, 0.1 mM ATP, and 5 mM DTT at 30 °C for 5 min, and the ability to synthesize DNA upon addition of [3H]dNTP was quantified. Insets, shown are the Coomassie Blue-stained 4–20% gradient SDS-polyacrylamide gel of the activity peak fraction. 150 μl of each fraction was trichloroacetic acid-precipitated and analyzed by SDS-PAGE.

for control IC, b and d appeared at 10 and 20 s, respectively (Fig. 7B). Based on the production of these fragments, the apparent elongation rates of isolated ICs in the absence of SSB were calculated as −35 nucleotides per second for IC-[δβ' χφ] and −177 nucleotides per second for the control IC. The elongation rate of IC-[δβ' χφ] was about 3-fold slower than that of control IC.

δβ' χφ Is Required For Full Extension of 8.6-kb M13 DNA Replication in the Absence of SSB—By having demonstrated that the lack of δβ' or δβ' χφ within elongation complex is accompanied by a decreased rate of DNA synthesis, we next examined whether δβ' affects the extent of M13Gori DNA synthesis. After a sufficient incubation time to complete a full circle of M13 DNA replication based on the observed elongation rate, the pattern and intensity of each fragment in the same lane was quantified using a PhosphorImager. We found that isolated IC-[δβ' χφ] was not able to complete 8.6-kb M13 DNA synthesis resulting in nearly half of the M13 DNA molecule remaining unreplicated (Fig. 7). The last fragment f (a size of 3.7 kb) was not visible even with the prolonged incubation time (Fig. 7A, lane 15 min). However, under the same experimental conditions, the control IC replicated the M13 DNA with full extension (8.6 kb) (Fig. 7B, lane 5 min). The comparison of ratios of early fragments (a, b, or d) and late fragments (e or f) of the two complexes, which was obtained by quantifying the intensity of DNA molecules corrected by its size, are shown in Table IV. After a sufficient incubation time enough to complete a full circle of M13 DNA replication, neither the intensity ratio nor the generated pattern of any given DNA fragments changed with further incubation (Fig. 7). At 10 min the ratios of each DNA fragment of IC-[δβ' χφ] were as follows: a and c (2.57), b (1.0), d (0.73), and e (0.08) (Fig. 7A, lane 10 min) and at 15 min were a and c (2.8), b (1.0), d (0.76), and e (0.05) (Fig. 7A, lane 15 min); those of control IC at 5 min were as follows: a and c (2.3), b (1.0), d (1.4), e (0.6), and f (0.13) (Fig. 7B, lane 5 min) and at 10 min were a and c (2.2), b (1.0), d (0.87), e (0.24), and f (0.06) (Fig. 7B, lane 10 min), respectively. Furthermore, we found that the ratio between the early fragment d (or b) and the...
late fragment e of IC-[δδ′ χψ] was ~10-fold lower as compared with the control IC (Table IV, see ratio between d and e). This ratio did not change with either an increased reaction time (5–20 min) or by an increased exposure (30 min to overnight). Thus, not only was the synthesis of the last fragment ψ not due to reassembly of dissociated initiation complexes, an ATP-dependent processivity factor was not required for elongation in IC-[δδ′ χψ]. Control IC, which is active upon addition of dADPNP, dCTP, dGTP, and dTTP, was not stimulated by addition of δδ′, χψ, δ′, or δ′ alone (data not shown). Overall, the results obtained by using dADPNP (Fig. 9) and dATP (Fig. 8) were comparable, and we concluded that the stimulatory effect of δ′ on elongation is not due to reassembly of dissociated initiation complexes, an ATP-dependent process, but due to a bono fide contribution of δ′ to the elongation phase of the holoenzyme-catalyzed reaction.

**DISCUSSION**

δ and δ′ are auxiliary subunits of DNA polymerase III holoenzyme required for the assembly of βε processivity factor onto the primed template for processive replication (17, 18). In the studies presented in this report, we showed that δ and δ′ are also parts of IC and participate in elongation by promoting the rate and extent of DNA chain elongation.

To generate reagents for these studies, we devised purification methods that yield homogenous, recombinant δ or δ′ subunit preparations free of trace of contaminating polymerases and nucleases.

Neither DnaX (πγ), which is related to δ′ in sequence (25), nor δ, which by itself is capable of unloading the βε clamp from DNA (22), could overcome the requirement for δ′ in reconstituting active DNA polymerase III holoenzyme. This suggests that δ′, which bridges DnaX and δ, has unique and constitutive functions. The requirement for δ′ was not overcome by δ′ alone since δ′ DNA, consistent with previous results indicating a key role for δ in contacting β within the clamp loader (γδδ′ χψ) (8). The biochemical requirement for δ′ or δ′ was confirmed by knocking out the structural gene for δ (holA) or δ′ (holB) in bacterial strains revealing the essential nature of both genes. Substitu-

**TABLE IV**

| Restriction fragment | IC-[δδ′ χψ] ratio<sup>a</sup> | IC ratio<sup>a</sup> |
|----------------------|-----------------------------|-------------------|
| a, c                 | 2.5 (±0.2)                  | 2.3 (±0.1)        |
| b                    | 1.0                         | 1.0               |
| d                    | 0.7 (±0.1)                  | 1.1 (±0.3)        |
| e                    | 0.05 (±0.01)                | 0.4 (±0.15)       |
| f                    | 0                           | 0.1 (±0.03)       |

<sup>a</sup> (intensity value/bp<sup>fragment</sup>/intensity value/bp<sup>fragment</sup>)

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Fig. 8. Activity of IC-[δδ′ χψ] is restored by additional δδ′ but not by ψ, δ, or δ′. Replication activity was determined by measuring the amount of total nucleotide incorporated into acid-insoluble DNA upon addition of [3H]dNTPs for the 5 min assay at 30 °C, 10 μl of isolated IC was added in the buffer containing 20 mM Hepes (pH 7.4), 0.2 mM ATP, 10 mM Mg(OAc)<sub>2</sub>, 5 mM DTT in a total 25-μl volume assay. A, replication activity of the IC-[δδ′ χψ] upon addition of the indicated amount of δ, δ′, χψ, δ′ χψ, δδ′, or δδ′ χψ, respectively. B, activity of the control IC that was formed with holoenzyme reconstituted using pol III, r, δ, δ′ and ψ (see “Experimental Procedures”) upon addition of the indicated amount of δδ′, δδ′ χψ, or χψ, respectively.
FIG. 9. The stimulatory effect of δ′ in elongation still remains with dADPNP in the absence of ATP. Replication activities of isolated ICs were determined in the presence of ATP (A) or in the absence of ATP (B and C) by using 48 μM dADPNP in place of dATP in A–C. 10 μl of each isolated ICs were added along with radioactive dNTPs at 30 °C in 20 mM Hepes (pH 7.4), 10 mM Mg(OAc)₂, 5 mM DTT in a total 25-μl volume. A, replication activities of isolated control IC (formed by use of pol III, τ complex containing χ-δ and β alone (open square) and the IC-[δ′ χ ψ]) for various times upon adding 1.2 pmol of δ′ (closed circle), χ (open triangle), ψ (open circle), or buffer (none, closed inverted triangle), in the presence of 0.2 mM ATP with dADPNP, [3H]dATP, dGTP, and dCTP. B, replication activities of isolated control IC alone (open square) and the IC-[δ′ χ ψ] for various times upon adding 1.2 pmol of δ′ (closed circle) in the absence of ATP with dADPNP, [3H]dTTP, dGTP, and dCTP. C, replication activity of IC-[δ′ χ ψ] upon adding various protein concentrations of δ′ (closed circle) or χ (open diamond) with dADPNP in the absence of ATP in a 20-min assay at 30 °C.

This analysis was used to confirm the specific removal of δ′ from the IC lacking both δ and δ′. Our system provides an elongation complex of pol III/βββ. The specific removal of δ′ from the IC permitted us to distinguish the requirements imposed on δ′ or δ′ χ ψ in elongation. Unlike δ′-IC, IC-[δ′ χ ψ] contained amounts of τ, α, and β that were comparable to those of τ-IC or n-IC. Since βββ cannot be loaded onto the DNA without δ′, the presence of the comparable amount of βββ in IC-[δ′ χ ψ] indicates that δ′ χ ψ is involved in the activity of the elongation complex.

To complete the replication of the 4.4-megabase E. coli genome within 40 min, the DNA polymerase III holoenzyme must synthesize DNA at a rate of about 1 kb/s. In vitro, replication assays have shown that native holoenzyme as well as reconstituted holoenzyme in the presence of SSB synthesize DNA at a rate of 500 nucleotides/s at 30 °C (50). We used an SSB-free system to test the requirements imposed on SSB synthesis activity of the elongation complex.

We showed that the lack of δ′ in elongation decreases the rate of DNA synthesis. Moreover, the full-length replication of the M13cont template was severely impaired curtail the extent of chain elongation. Only a small fraction of enzyme-DNA complex was able to replicate continuously to produce a long DNA product (>3–4 kb) in the absence of δ′ χ ψ resulting the decreased total sum of overall DNA synthesis in IC-[δ′ χ ψ]. Presumably, the lack of δ′ χ ψ in elongation causes the polymerase to stall on the DNA or results in a premature dissociation from the DNA. These results, together with the observed decrease in the rate of elongation (3-fold), account for the observed significant decrease (8–10-fold) in the overall amount of DNA synthesis as measured using the holoenzyme activity assay.

We showed that the adding back δ′ but not χ δ′, δ′ δ′, or δ′ δ′ restored the diminished activity of IC-[δ′ χ ψ] in the absence of SSB. This indicates that the observed results are mainly due to the requirement of δ′ together, but not δ′ χ, δ′, or δ′ alone. However, a critical in vitro role for χ ψ is not ruled out by the results of these studies. We already know that a χ-SSB interaction is critical for replication of DNA at physiological levels of salt and for maintaining a DNA-X-δ′ at physiological protein levels (24, 30, 40). It is possible that the effect of added δ′ is mediated by an assembly mechanism that is different from that of the cooperative DnaX

The results obtained using a reconstituted enzyme system were compared ICs generated via use of the native versus reconstituted forms of the holoenzyme. n-IC was physically and functionally similar to τ-IC, an IC generated using a reconstituted enzyme formed with the τ-complex along with other reconstituted components. Both n-IC and τ-IC contained δ and δ′, as well as each of the other subunits of the τ-complex, the processivity factor, and the polymerase core. These two complexes exhibited comparable DNA synthesis activities upon addition of dNTPs. However, quite different results were obtained when we attempted to generate an IC-utilizing holoenzyme reconstituted with the γ-complex. Not only the resultant complex did not contain any of the subunits of the γ-complex (γ, δ, δ′, χ, or ψ) but also the amount of pol III incorporated within this IC was significantly less than those of τ-IC or n-IC. These findings suggest that although the γ-complex plays a catalytic role in βββ loading, the βββ and the pol III core are more efficiently introduced onto DNA when the IC has a τ-containing DnaX complex (τ5δδ′ χ ψ or τ5δδ′ χ ψ) (9). Moreover, the overall amount DNA synthesis per unit of polymerase (pol III), was notably less than that of τ-IC or n-IC. This indicates a potential role of the missing subunits in γ-IC in a downstream stage of the holoenzyme reaction-elongation. It has been reported that the τ subunit has a role in stabilizing pol III on the DNA and facilitating DNA elongation (48, 49).
complex assembly promoted by $\gamma\psi$. By using a dADPNP, an analog that cannot support initiation complex formation, in place of dATP to support elongation, we showed that this stimulatory effect of $\delta\delta'$ on the rate and extent of elongation mediated by IC-[\delta\delta'] does not require ATP. This suggests that the stimulatory effects of $\delta\delta'$ are not due to the re-formation of dissociated initiation complexes, a reaction that requires ATP hydrolysis. Overall, these findings provide evidence that $\delta\delta'$ participates in elongation.

Because $\delta$ and $\delta'$ are a part of the DNAx complex, our findings can be further extended to suggest a participatory role of the DNAx complex in elongation. This adds to the role of the DNAx complex in coupling the DNA polymerase III holoenzyme to the DNA helicase at the fork and in coupling the leading and lagging strand polymerases in which the function of DNAx complex in assembly promoted by IC-$\delta\delta'$ does not require ATP. This suggests that the function of DnaX participates in elongation.

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