The genes for the polymerase core (αεθ) of the DNA polymerase III holoenzyme map to widely separated loci on the Escherichia coli chromosome. To enable efficient overproduction and in vivo assembly of DNA polymerase III core, artificial operons containing the three structural genes, dnaE, dnaQ, and holE, were placed in an expression plasmid. The proteins α, αε and αεθ were overexpressed and assembled in E. coli and purified to homogeneity. The three purified polymerases had a similar specific activity of about 6.0 × 10^6 units/mg in a gap-filling assay. Kinetics studies showed that neither ε nor θ influenced the Km of α for deoxynucleotide triphosphate and only slightly decreased the Km of α for DNA, although ε was absolutely required for maximal DNA synthesis. The rate of DNA synthesis by α-reconstituted holoenzyme using τ complex was about 5-fold less than that of αε or αεθ-reconstituted holoenzyme as determined by a gel analysis. The processivity of α-reconstituted holoenzyme was very similar to that of αεθ-reconstituted holoenzyme when τ complex was used as a clamp loader.

The DNA polymerase III core (pol III)² of the DNA polymerase III holoenzyme is a heterotrimer, composed of α, ε, and θ subunits of 129,900, 27,500, and 8,700 daltons, respectively (McHenry and Crow, 1979). The subunits of pol III are expressed from genes located at separate sites on the Escherichia coli chromosome; α is encoded by dnaE (Gefter et al., 1971; Welch and McHenry, 1982), ε by dnaQ (Horiuchi et al., 1981; Sheuermann et al., 1983), and θ by holE (Studwell-Vaughan and O'Donnell, 1993; Carter et al., 1993). The three subunits form a very stable complex at a ratio of 1:1:1. α binds ε which binds θ, but a direct α-ε-θ contact has not been observed (Studwell-Vaughan and O'Donnell, 1993).

Individual subunits of pol III have been overexpressed, purified, and characterized. The α subunit contains catalytic polymerase activity and synthesizes DNA at a rate of approximately 10 nucleotides/s (Maki and Kornberg, 1987, Maki and Kornberg, 1985). The ε subunit (the dnaQ product) contains 3' → 5' exonuclease activity for the proofreading function of DNA replication (Sheuermann and Echols, 1984); thus, dnaQ (mutD) has a strong mutator phenotype (DiFrancesco et al., 1984). The function of the θ subunit in DNA replication is unclear.

Pol III is dimerized via the interaction between the τ and α subunits, resulting in the formation of a dimeric polymerase that enables the coordinated synthesis of the leading and lagging strands (McHenry, 1982; Studwendl-Vaughan and O'Donnell, 1991). The C-terminal region of α binds to a dimer with a high affinity (Kd = 70 pm) (Kim and McHenry, 1996). Pol III itself is distributive but becomes a processive and rapid polymerase on a primed template with other accessory subunits (Fay et al., 1981). The DnaX complex (τδδ'ψ or γδδ'ψ) loads the β sliding clamp onto the primed template by coupling ATP hydrolysis (Dallmann and McHenry, 1995; Onrust et al., 1995). The β sliding clamp provides pol III with high processivity by tethering it to the template (LaDuca et al., 1986; Stukenberg et al., 1991).

Each subunit of pol IIIL works cooperatively and stimulates the activity of other subunits. For instance, the α subunit can stimulate the exonuclease activity of the ε subunit 10-80-fold by increasing the affinity of ε for the 3'-hydroxyl terminus (Maki and Kornberg, 1987). The ε exonuclease activity is also slightly stimulated by the θ subunit (Studwell-Vaughan and O'Donnell, 1993). Additionally, ε induces a 3-fold increase in the polymerase activity of the α subunit (Maki and Kornberg, 1987). Thus, three pol III subunits are functionally cooperative. In fact, most DNA polymerases contain separate domains for the polymerase and exonuclease activities in a single polypeptide, suggesting that the two activities are interactive (Blanco et al., 1991).

In this present study, we constructed artificial operons that overexpress either α, αε, or αεθ complexes assembled in vivo and purified them to homogeneity without the denaturation-renaturation step required for the purification of ε due to its insolubility. The three purified polymerases were characterized, and their function and kinetics in DNA replication were compared.

**Experimental Procedures**

Strains—E. coli strains HB101 (F', recA13, ara 14, proA2, lacY1, galK2, rpsL20, xy15), J109 ( recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lambda-), IacP230 (Del), and MC1061 (araD139, ara LEU769, galU, galK, lac 174 (DEL), hsdR2, mcrB1, rpsL) were used for plasmid propagation and protein expression.

Cell Growth and Induction—E. coli strains containing overexpressing plasmids were grown in 200 liters of P medium (1.5% yeast extract, 1% peptone, 1.2% K2HPO4, 0.12% KH2PO4, 1.0% glucose) plus 50 μg/ml ampicillin at 37°C. Cells were induced by isopropyl-β-d-thiogalactoside (1 mM final concentration) at A600 = 1.0. After 4.5 h (2.5 h for α expression), cells were harvested by centrifugation, resuspended in 1:1, w/v in 50 mM Tris-HCl (pH 7.5) and 10% sucrose, and immediately frozen in liquid N2.

Chromatographic Supports—Bio-Rad 70 columns were purchased from Bio-Rad. Sephacryl-300 HR resins were from Pharmacia. Toyopearl-560 x 50 columns were obtained from Tosohaa.

Proteins—The τ complex (τδδ'ψ) and γ complex (γδδ'ψ) were
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**Fig. 1. Construction of plasmids to overexpress αe and pol III.**
Plasmids were constructed as described under "Experimental Procedures." The backbone of the vectors was derived from pJC1, an HIV nucleocapsid (NC)-overexpressing plasmid (You and McHenry, 1993), which has a tac promoter, a replication origin (ori), αe gene, two transcriptional terminators (T1 and T2). (Brosius et al., 1981), and the structural gene for β-lactamase (ampβ). S/D indicates a Shine-Dalgarno site. The backbone of the vectors was derived from pJC1, an HIV nucleocapsid (NC)-overexpressing plasmid (You and McHenry, 1993), which has a tac promoter, a replication origin (ori), αe gene, two transcriptional terminators (T1 and T2). (Brosius et al., 1981), and the structural gene for β-lactamase (ampβ). S/D indicates a Shine-Dalgarno site.

reconstituted and purified as described (Dallmann and McHenry, 1995). Sequenase version 2.0 and Dnase I were obtained from United States Biochemical Corp. Terminal deoxynucleotidyltransferase was prepared as described (Johanson and McHenry, 1980). Buffers—Buffer I is 50 mM imidazole (pH 6.5), 1 mM EDTA, 50 mM Tris-HCl (pH 7.5), 20% glycerol; buffer A is 20 mM potassium phosphate (pH 6.5), 1 mM EDTA, 50 mM Tris-HCl, and 25% glycerol; 30% A.S. buffer is 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM Tris-HCl, and 175 g of ammonium sulfate added to 1 liter (30% saturation at 4°C); 15% A.S. buffer is 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM Tris-HCl, and 84 g of ammonium sulfate added to 1 liter (15% saturation at 4°C); buffer B is 50 mM Hepes (pH 7.5), 20% glycerol (v/v), 10 mM EDTA, 0.1 mM EDTA, 100 mM potassium glutamate, and 5 mM magnesium acetate; buffer C is 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM Tris-HCl, and 30% glycerol; buffer E is 50 mM Tris-HCl (pH 7.5), 5% glycerol, and 1 mM EDTA; enzyme dilution buffer (EDB) is 50 mM Hepes (pH 7.5), 0.02% Nonidet P-40, 100 µg/ml BSA, 20% glycerol (v/v), 10 mM EDTA, and 100 mM potassium glutamate; buffer TE is 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Oligonucleotides—Two primers used to amplify the dnaQ gene were synthesized on a Biosearch 8600 DNA synthesizer. The 5′-primer is 5′-GGGG AGATCT AGGAGG TTT AAA TATG AGC ACT GC (AGGAGG), and the 5′-primer is 5′-CCCC CCC CAA GCT TCA CCA AGT GGC GCG GCC CTC ATG TAT CTT CAG AAG (AGGAGG), and the 5′-primer is 5′-CCCC CCC CAA GCT TCA CCA AGT GGC GCG GCC CTC ATG TAT CTT CAG AAG (50-mer). These two oligonucleotides were purified by DES2 column chromatography as described (Hagerman, 1985). Another four oligonucleotides (5′-AGGG AGATCT GAT CAA TCA TCA TAA TAA TGA GAT CCGCAA TTA CAC GCC-3′ (48-mer), and the 5′-primer is 5′-CCCC CCC CAA GCT TCA CCA AGT GGC GCG GCC CTC ATG TAT CTT CAG AAG (50-mer). These two oligonucleotides were purified by DES2 column chromatography as described (Hagerman, 1985). Another four oligonucleotides (5′-AGGG AGATCT GAT CAA TCA TCA TAA TAA TGA GAT CCGCAA TTA CAC GCC-3′ (48-mer), and the 5′-primer is 5′-CCCC CCC CAA GCT TCA CCA AGT GGC GCG GCC CTC ATG TAT CTT CAG AAG (50-mer). These two oligonucleotides were purified by DES2 column chromatography as described (Hagerman, 1985).

Fig. 2. Overexpression of αe, α, and θ from pHN4. Total cell proteins before and after induction of E. coli strain HB101 (pHN4) were prepared as described (Kim and McHenry, 1996), and 20 µl of each sample was loaded on a 10-20% gradient SDS-polyacrylamide gel. Proteins were separated at a constant 65 V overnight. The gel was stained with Comassie Brilliant Blue overnight and destained in a solution of 10% methanol and 10% acetic acid. Lane 1, protein markers; lane 2, uninduced total cell proteins; lane 3, induced total cell proteins.

yielding a matched base pair (C) and three mismatched base pairs (G, A, T) to the template (G).

Construction of the Artificial Operon of Pol III Core—The dnaQ gene of pNS121 (Scheuermann et al., 1983) was amplified using two primers, the 5′-primer contains a 22-nucleotide sequence complementary to dnaQ, a Shine-Dalgarno site (AGGAGG), and a BglII restriction enzyme site; the 3′-primer has a 16-nucleotide sequence complementary to dnaQ and three cloning sites (PstI, DraI, and HindIII). The polymerase chain reaction was conducted as described (Saiki et al., 1988). The polymerase chain reaction products of dnaQ were digested with BglII and HindIII and ligated to the large fragment of pJC1 (You and McHenry, 1993) digested at the same restriction sites (Fig. 1). The resulting plasmid was named pHN1, an αe overexpressing plasmid. The plasmid pHN3, an αe overexpressing plasmid, was generated by ligation of the PstI-DraII fragment of pHN1 and the same restriction enzyme-digested fragment of pOPPA50-42a, an αe overexpressing plasmid (Tomasiewicz, 1991). Finally, the hoE gene, encoding the θ subunit, from pHN100 (Carter et al., 1993) was inserted into pHN3 at the PstI site between dnaQ and dnaE to generate plasmid pHN4, which overexpressed pol III core (coel) complex. Each gene of pol III in this plasmid contains its own Shine-Dalgarno sequence in front of a start codon (ATG) with an AT-rich, 9-nucleotide spacer.

Determination of Molar Extinction Coefficients—The extinction coefficients of the α subunit, α complex, and pol III core (coel) complex at ε290 were 99,920, 112,370, and 123,000 liters mol⁻¹ cm⁻¹, respectively, as determined by the method of Edelhoch (1967). Proteins were dialyzed against buffer E overnight, and their extinction coefficients were determined in buffer E, in the presence or absence of 6 M guanidine hydrochloride. Spectra of the three polymerases were measured on a Hewlett-Packard 8450Z diode array spectrophotometer between 240 and 340 nm. Extinction coefficients of denatured proteins were calculated from the number of tryptophan and tyrosine residues in each protein (Edelhoch, 1967) and corrected by the ratio of the absorbances of the native proteins to the absorbances of the proteins in 6 M guanidine hydrochloride.

Gap-filling Polymerase Assay—This assay was performed by a modification of the method of McHenry and Crow (1979) and used to detect enzyme activity during protein purification. The reaction was initiated by the addition of enzyme to a 25-µl solution containing four dNTPs (100 cpnm⁻¹ dNTPs), 10 mM MgCl₂, and 5 µg of activated calf thymus DNA in 50 mM Hepes (pH 7.5), 10 mM DTT, 200 mg/ml BSA, 0.02% Nonidet P-40, and 20% glycerol and incubated at 30 °C for 5 min. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dNTPs per min at 30 °C.
Preparation of Activated Calf Thymus DNA—Calf thymus DNA (100 mg) was dissolved in 50 ml of 20 mM KCl with stirring overnight at 4 °C. The dissolved DNA was treated with 0.4 μg of DNAse I (1 mg/ml in 1 mM CaCl₂ and 50% glycerol) per 10 mg DNA in a reaction mix containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 50 μg/ml BSA at 37 °C for 20 min. DNase I was inactivated at 65 °C for 10 min, followed by phenol: chloroform extraction, and an additional chloroform extraction. The DNA was precipitated by addition of 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2) (−20 °C overnight). DNA samples were centrifuged at 10,000 × g for 35 min and rinsed with 70% ethanol twice. The DNA pellet was initially dissolved in 30 ml of buffer TE and dialyzed against 2 liters of buffer TE for 4 h with one buffer change. The DNA concentration was determined spectrophotometrically at 260 nm (17 A₂₆₀ = 1 mg/ml) and adjusted to 5 mg/ml final concentration by dilution with buffer TE.

Determination of Steady-state Kinetic Parameters—Kinetic parameters for dNTPs and activated DNA of the three polymerases (α, αε, αευ) were determined in the gap-filling polymerase assay described above in the presence of 67 fmol of each polymerase at 30 °C for various times. dNTPs were titrated in the presence of 605 μM activated calf thymus DNA (as nucleotide), or activated DNA was titrated in the presence of 60 μM dNTPs to determine an initial velocity at each substrate concentration. The Kₘ and Vₘₐₓ were calculated from Lineweaver-Burk plots or by nonlinear least squares curve fitting (Kaleidagraph 3.0.1 software) to the equation ν₀ = Vₘₐₓ [S]/(Kₘ + [S]), where ν₀ is initial velocity at a given substrate concentration, and S is substrate concentration. The kinetic parameters (Kₘ and Vₘₐₓ) were determined by these two different methods to agree to within ±5%.

Preparation of Primed M13ori DNA—RNA-primed and SSB-coated M13ori DNA was prepared (Fay et al., 1981). A reaction mix (1 ml) containing M13ori DNA (1 μmol as nucleotide), SSB (1.6 mg), DnaG primase (87,500 units), four NTPs (each 0.5 mM), and magnesium acetate (10 mM) in buffer B was incubated at 30 °C for 30 min and applied to a Bio-Gel A-5m column (1 × 25 cm) equilibrated with buffer B at 4 °C. Primed M13ori DNA was eluted with 40 ml of buffer B (flow rate 125 μl/min) and detected by assaying with holoenzyme subunits and dNTPs (Dallmann et al., 1995). The peak fractions (total 1.8 ml) that incorporate more than 40 pmol of nucleotides in a 25-μl assay using 1 μl of each fraction were combined (83% yield based on replication assay).

Analysis of DNA Elongation Rate—The DNA synthesis rate of the holoenzyme-like activity reconstituted with three polymerases (α, αε, αευ) and τ complex and β was determined by a modification of the method of Fay et al. (1981). A reaction mix (50 μl) containing RNA-primed, SSB-coated M13ori DNA template (480 fmol as a circle), τ ori DNA template (480 fmol as a circle), DnaG primase (87,500 units), four NTPs (each 0.5 mM), and magnesium acetate (10 mM) was incubated at 30 °C for 10 min and 5% NaCl standards. The actual yield of pol III from S-300 was 18 mg as determined using the extinction coefficient. Thus, the true specific activity of pure pol III is 5.9 × 10⁶ units/mg.

| Table I Purification of pol III |
|--------------------------------|
| Fraction | Total protein (mg) | Total units | Specific activity (units/mg) | Yield (%) |
| I. Lysate | 15.600 | 2.9 | 19 | 100 |
| II. Ammonium sulfate | 945 | 1.9 | 201 | 65 |
| III. BioRex-70 | 98 | 1.3 | 1,360 | 45 |
| IV. S-300 | 43 | 1.0 | 2,500 | 34 |

*For purposes of convenient comparison, the protein concentrations reported were determined by the method of Bradford (1976). The actual yield of pol III from S-300 was 18 mg as determined using the extinction coefficient. Thus, the true specific activity of pure pol III is 5.9 × 10⁶ units/mg.

Fig. 3. Chromatographic purification of pol III. A, Bio-Rex 70 column profile. The first 20 fractions are flow-through, and the following 10 fractions are column fractions. B, Sephacryl S-300 column profile. A total of 80 fractions (1.375 ml each) were collected. Polymerase activity (●) and protein (●) were assayed as described under "Experimental Procedures." Conductivity (●) is reported relative to NaCl standards. B, SDS-polyacrylamide gel electrophoresis of Bio-Rex 70 fractions (70 μl) and Fr II (50 μl, load lane). Fractions were analyzed by 10–20% gradient SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue to visualize proteins. The protein content of applied peak fractions was about 20 μg. Numbers above the gel are column fractions.
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complex (1.64 pmol as εab subunit), β subunit (2.26 pmol as dimer), and 200 μM ATP in enzyme dilution buffer was incubated at 30 °C for 5 min with 1 pmol of α, ε, or αε to allow formation of an initiation complex. The reaction was then placed in a 22 °C water bath for 5 min to permit thermal equilibration and started by the addition of 6 μl of dNTPs (each 0.8 mM) at 22 °C. All reaction mixtures were initially made in a batch, and a 50-μl sample was removed at the indicated time and quenched in 200 μl of ethanol and 5 μl of 4 m NaCl in a dry ice/ethanol bath. DNA samples were precipitated at −80 °C overnight, spun at 15,000 × g for 45 min at 4 °C, and resuspended in 26 μl of H2O. The DNA was digested with BbvI in a 30-μl volume at 37 °C for 1 h, loaded on an 8% native polyacrylamide gel (1.5 × 25 × 15 cm), and run at 100 V overnight. The gel was stained with ethidium bromide (25 μg/ml) solution for 30 min and destained in a 1 mM MgSO4 solution for 10 min to visualize DNA fragments with a UV illuminator.

Other Methods—Protein concentration during protein purification was determined by the method of Bradford (1976). Protein concentration of all purified proteins was determined using the extinction coefficient. SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (1970).

RESULTS

We constructed artificial operons to overexpress pol III subunit complexes assembled in vivo. The dnaE, dnaQ, and holE genes were inserted into a vector with a tac promoter to produce the αεθ complex, whereas dnaE and dnaQ were inserted to obtain a plasmid expressing the αε complex (Fig. 1). Three polymerases (α, ε, or αεθ) were overexpressed and purified to 99% homogeneity from overexpressing E. coli strains. All steps of preparation were performed at 0–4 °C unless noted otherwise.

**TABLE I**

| Fraction | Total protein (mg) | Total units (× 10^3) | Specific activity (units/mg) | Yield (%)
|----------|--------------------|----------------------|----------------------------|----------
| I. Lysate| 40,390             | 18                   | 100                        | 44       |
| II. Ammonium sulfate | 2,418              | 9                    | 50                         | 370      |
| III. Bio-Rex 70 | 367                | 6.4                  | 35                         | 1,731    |
| IV. S-300 | 122               | 3.6                  | 20                         | 2,860    |

<sup>a</sup> The yield determined using the extinction coefficient was 55 mg.
<sup>b</sup> Thus, the true specific activity of αε is 6.3 × 10^6 units/mg.

**TABLE II**

| Fraction | Total protein (mg) | Total units (× 10^3) | Specific activity (units/mg) | Yield (%)
|----------|--------------------|----------------------|----------------------------|----------
| I. Lysate| 23,904             | 6.4                  | 100                        | 26       |
| II. Ammonium sulfate | 8,700              | 8.5                  | 132                        | 97       |
| III. Bio-Rex 70 | 217                | 3.7                  | 58                         | 1,687    |
| IV. Phenyl-650M | 66                 | 1.5                  | 23                         | 2,216    |
| V. S-300 | 32                 | 0.8                  | 12                         | 2,546    |
| VI. Heparin | 17^a               | 0.5                  | 8                          | 2,941^a |

<sup>a</sup> The yield determined using the extinction coefficient was 9.5 mg.
<sup>b</sup> Thus, the true specific activity of α is 6.6 × 10^6 units/mg.

**TABLE III**

| Fraction | Total protein (mg) | Total units (× 10^3) | Specific activity (units/mg) | Yield (%)
|----------|--------------------|----------------------|----------------------------|----------
| I. Lysate| 40,390             | 18                   | 100                        | 44       |
| II. Ammonium sulfate | 2,418              | 9                    | 50                         | 370      |
| III. Bio-Rex 70 | 367                | 6.4                  | 35                         | 1,731    |
| IV. S-300 | 122               | 3.6                  | 20                         | 2,860    |

<sup>a</sup> The yield determined using the extinction coefficient was 55 mg.
<sup>b</sup> Thus, the true specific activity of αεθ is 6.3 × 10^6 units/mg.

**Fig. 4.** Chromatographic purification of α, A, Toyopearl phenyl-650M column profile. A total of 80 fractions (7.5 ml each) were collected. Polymerase activity (●) and protein (▲) were assayed as described under “Experimental Procedures.” Conductivity (▲) is given as the saturation percentage of ammonium sulfate at 4 °C. B, SDS-polyacrylamide gel electrophoresis of Toyopearl phenyl-650M fractions (35 μl) and Fr III (40 μg, load lane). Fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue. Applied peak fractions in this gel contain about 30 μg of protein. Numbers above the gel are column fractions. C, heparin-Sepharose column profile. A total of 80 fractions (3 ml each) were collected. Polymerase activity (●) and protein (▲) were assayed as described under “Experimental Procedures.” Conductivity (▲) is given as mM NaCl. D, SDS-polyacrylamide gel electrophoresis of heparin-Sepharose fractions (30 μl) and Fr V (40 μg, load lane). Fractions were analyzed by 10% SDS-polyacrylamide gel electrophoresis and the gel was stained with Coomassie Blue. Applied peak fractions contain about 37 μg. Numbers above the gel are column fractions.
scan (Fig. 2, lane 3). The presence of free $\alpha$ or $\alpha\varepsilon$ would not be expected since $\alpha$ was the limiting subunit. The extra $\varepsilon$ and $\theta$ subunits formed a soluble, separable complex (data not shown) through a direct interaction; excess $\varepsilon$ itself was insoluble.

Cell Lysis and Ammonium Sulfate Precipitation—Frozen cells (180 g) were thawed and lysed (2 mg of lysozyme per g of cells) to prepare cell lysates (Fr I) as described (Cull and McHenry, 1995). Initially, 0.226 g of ammonium sulfate (40% saturation at 0°C) for each ml of cell lysate was added, followed by two sequential backwashes with 0.200 and 0.170 g of ammonium sulfate added to each ml as described (Cull and McHenry, 1995). The final ammonium sulfate precipitate was resuspended in buffer I and applied to a 190-ml pre-equilibrated Bio-Rex 70 column (5.75 × 7.32 cm) (Fig. 3A). The pool (Fr III, 965 ml, Table II) of fractions 37 to 78 was dissolved in buffer A (2.0 ml) and loaded onto the column. The activity eluted at fractions 46–52, so that trace contaminants could be detected. However, when 10–20 μg of this pool was loaded and resolved on a gel, no contamination was detected (Fig. 5). The overall yield of pol III in the purification was 34% (Table I).

**Purification of $\alpha\varepsilon$ Complex**

Cell Lysis and Ammonium Sulfate Precipitation—E. coli HB101 containing pHN3 produced the $\alpha\varepsilon$ complex at a level of 2% $\alpha$ and 20% $\varepsilon$ of total proteins (data not shown). Cell lysis (286 g of cells) and ammonium sulfate precipitation were performed under the same conditions as described for pol III purification except that the 0.170 backwash was skipped because it resulted in solubilization of a significant amount of $\alpha\varepsilon$. $\alpha$ and $\varepsilon$ formed a tight complex at a 1:1 ratio, and the excess insoluble $\varepsilon$ subunits sedimented with cell debris (data not shown).

Bio-Rex 70 and Sephacryl S-300 Chromatography—A 280-ml Bio-Rex 70 column (2.7 × 49.5 cm) was prepared, and proteins were loaded onto the equilibrated column as described for pol III. The column was washed with buffer I + 75 mM NaCl, and proteins were eluted with a 10-column volume buffer I + 25 mM NaCl to buffer I + 300 mM NaCl gradient. The $\alpha\varepsilon$ complex started to elute at a conductivity of buffer I + 125 mM NaCl (Fig. 3A). The pool of peak fractions (72-98) (Fr III, 490 ml, Table I) was precipitated by the addition of an equal volume of saturated ammonium sulfate solution. The purity of pol III after this column was more than 80% based on densitometric scan of an SDS gel (Fig. 3B). All three subunits ($\alpha$, $\varepsilon$, and $\theta$) eluted in a constant ratio across the peak (Fig. 3B).

Sephacryl S-300 HR Gel Filtration Chromatography—The protein pellet obtained from ammonium sulfate precipitation of Fr III was dissolved in buffer A + 100 mM KCl. The resulting protein solution (2 ml) was loaded onto a 110-ml, equilibrated Sephacryl S-300 column (1.5 × 62.3 cm), and 80 fractions were collected at a flow rate of 0.1-column volume/h. Pol III eluted at fractions 44–54 (Fig. 3C), resulting in Fr IV (15.1 ml, Table I). Individual fractions (50 μl) were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3D). The gel was overloaded (about 175 μg at fractions 46–52), so that trace contaminants could be detected. However, when 10–20 μg of this pool was loaded and resolved on a gel, no contamination was detected (Fig. 5). The overall yield of pol III in the purification was 34% (Table I).

**TABLE IV**

| Substrate | $\alpha\varepsilon$, pol III | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------|-----------------------------|-------|-----------|--------------|
| dNTP      | $\alpha$                    | 22 ± 1| 12.1 ± 1.2| 5.5 × 10⁶    |
|           | $\alpha\varepsilon$         | 21 ± 2| 19.8 ± 2.5| 9.4 × 10⁶    |
|           | $\alpha\varepsilon\theta$   | 24 ± 1| 26.9 ± 2.7| 1.1 × 10⁷    |
| DNA       | $\alpha$                    | 100 ± 5| 11.9 ± 1.0| 1.2 × 10⁶    |
|           | $\alpha\varepsilon$         | 85 ± 3| 21.0 ± 3.3| 2.5 × 10⁶    |
|           | $\alpha\varepsilon\theta$   | 55 ± 5| 21.6 ± 3.5| 3.9 × 10⁶    |

Data were derived from a Lineweaver-Burk plot and nonlinear curve fitting program and represent the average of three separate experiments. DNA concentration is given as nucleotide. ~150 μM nucleotide is equivalent to 1 μM 3’-hydroxyl end of activated DNA.
Purification of the α Subunit

Cell Lysis and Ammonium Sulfate Precipitation—E. coli MC1061 containing pOPPA50-4a2 (Tomasiewicz, 1991) was used to express the α subunit to ~5% of total cell proteins (data not shown). Cell lysis (239 g of cells) was performed as described for pol III purification. The α subunit alone was much more soluble in ammonium sulfate than the other two polymerase forms; it did not precipitate significantly under conditions described for pol III or αε complex purification. Initially, 0.164 g of ammonium sulfate (30% saturation at 0°C) was added to each ml of Fr I lysate. Insoluble protein was removed by centrifugation (23,300 × g at 0°C for 1 h). The supernatant, containing α, was adjusted to a final concentration of 0.291 g/ml ammonium sulfate (50% saturation at 0°C). Precipitates (Fr II) were collected by centrifugation as described above.

Bio-Rex 70 Chromatography—A 450-ml Bio-Rex 70 column (5.75 × 17.3 cm) was used in an identical manner as described for pol III. The α subunit eluted at the same conductivity as the αε and αεθ complexes (data not shown). Fractions 78–93 (Fr III, 445 ml, Table III) were pooled and precipitated by the addition of an equal volume of saturated ammonium sulfate solution.

Toyopearl Phenyl-650M Hydrophobic Chromatography—Although α was purified to near-homogeneity from the Bio-Rex column, it was less pure than pol III or αε. Thus, additional chromatographic steps were required. Ammonium sulfate-prefractionated Fr III was dissolved in 30% A.S. buffer and loaded onto a pre-equilibrated hydrophobic Toyopearl phenyl-650M column (60 ml, 1.5 × 36 cm). The column was washed with 1-column volume of 30% A.S. buffer followed by 2-column volumes of 15% A.S. buffer. Proteins were eluted with a 10-column volume 15–0% ammonium sulfate gradient at a flow rate of 0.84-column volume per h. The α subunit eluted at 8–6% saturating ammonium sulfate (Fig. 4, A and B). Fractions 42–60 were combined, resulting in Fr IV (150 ml, Table IIII). The pool of fractions was precipitated by the addition of an equal volume of saturated ammonium sulfate.

Sephacryl S-300 Chromatography—Gel filtration chromatography to remove aggregated proteins as well as to purify α further was carried out as for αε and αεθ. Ammonium sulfate-prefractionated Fr IV was dissolved in 2 ml of buffer A + 100 mM KCl and loaded onto a Sephacryl S-300 gel filtration column (160 ml, 1.5 × 90 cm). α eluted at fractions 46–52. The pool (Fr V, 13.5 ml) of this column was nearly pure, but some contamination was detected when more than 75 μg of protein was loaded on a gel (data not shown).

Heparin-Sepharose Chromatography—We conducted another chromatographic step to remove all trace contaminants. Fr V was dialyzed against buffer I + 10 mM NaCl and loaded onto a 19-ml heparin-Sepharose column (0.75 × 44 cm) equilibrated with buffer I + 10 mM NaCl. The column was washed with 2-column volumes of buffer I + 10 mM NaCl and eluted with 10-column volumes of gradient (buffer I + 10–300 mM NaCl) at a flow rate of 1-column volume per h. Fractions 58–62 (Fr VI, 15 ml, Table IIII) were combined. The α subunit eluted at a conductivity of buffer I + 150 mM NaCl (Fr, C and D).

SDS-Polyacrylamide Gel Electrophoresis of Purified Polymerase Forms—The three purified pol III polymerases (each 10 μg) were analyzed by SDS-gel electrophoresis (Fig. 5). Based on densitometric scan, all pol III polymerases were purified to 99% homogeneity. Stoichiometries of αε and αεθ were determined by laser densitometry of complexes subjected to SDS-polyacrylamide gel electrophoresis, stained with Coomassie Blue, and corrected for molecular weight. The stoichiometry of the three pol III subunits was 1.0:1.1:0.9 (αε:ε:θ). In the αε preparation, the ratio was 1.0:1.1 (α:ε).

Steady-state Kinetics of Polymerases in Gap-filling Assay—To examine and compare the kinetic properties of the three polymerase forms, the Km and Kcat values for dNTPs or activated DNA substrates (Table IV) were calculated by an iterative fit to the equation ~max/([S])/(Km+[S]) as described under “Experimental Procedures” (Fig. 6) and from Lineweaver-Burk plots. The Kcat was calculated from the equation Kcat = Vmax/Ei, where Ei is total enzyme concentration. The Km of all three polymerase forms for dNTPs was in the range of 21–24 μM. The Kcat value (~12 s−1) of α was 2-fold less than that of the other two polymerase forms (Table IV). The Km of all three polymerase forms for activated calf thymus DNA was very similar, although Km for pol III was slightly lower. Because activated calf thymus DNA is a heterogeneous template, Km values were defined in terms of total nucleotide concentration, permitting a relative comparison. The Kcat/Km of pol III for both dNTP and DNA was about 2-fold higher than that of α alone, indicating that ε and θ made a modest contribution to the gap-filling polymerase activity of α. The 3′-OH concentration of activated DNA was estimated from the average size of DNA fragments determined by denaturing gel electrophoresis and dNTPs incorporated between gaps, −150 μM as nucleotide was equivalent to 1 μM of 3′-OH.

Primer Extension from Mismatched 3′ Ends by Holoenzymes Reconstituted with α, αε, and αεθ—The α subunit itself does not have proofreading activity to remove misincorporated bases during replication. We asked whether α-reconstituted holoenzyme extends nucleotides further from the mispaired 3′ end or, instead, pauses in DNA elongation when an incorrect nucleotide is incorporated. Using four oligonucleotides annealed to M13G tide is incorporated. Using four oligonucleotides annealed to M13G.
ability of three highly purified polymerase forms allowed us to study the effect of 
and 
 on DNA synthesis rates by reconstituted holoenzyme. Initiation complexes were formed using saturating levels of either 
, 
, or 
 as described under "Experimental Procedures." Reactions were started by the addition of dNTPs at 22 °C (Fig. 8A). The DNA fragments generated from Bbvl digestion are a (900 bp), b (175 bp), c (690 bp), d (1739 bp), e (611 bp), f (1154 bp), g (410 bp), h (2000 bp), i (688 bp), and j (256 bp) in the order produced. Fragment d appeared at 2 min for 
-reconstituted holoenzyme and at 30 s for both 
- and 
-reconstituted holoenzymes, and fragment h appeared at 5 min for 
-reconstituted holoenzyme and at 1 min for 
- and 
-reconstituted holoenzymes (Fig. 8B). Based on the production of these fragments, the elongation rates of reconstituted holoenzymes at 22 °C were calculated as 28 ± 2, 126 ± 4, and 126 ± 4 nucleotides/s for 
-, 
-, and 
-reconstituted holoenzymes, respectively. The elongation rate of 
-reconstituted holoenzyme was approximately 5-fold slower than that of 
- and 
-reconstituted holoenzymes.

Processivity of the 
-Reconstituted Holoenzyme Using 
Com-
A cholesterol reconstituted holoenzyme complex was used (Fig. 9, lanes 2, 4, 6, and 8) or 1 pmol of α (lanes 3, 5, 7, and 9) to primed M13Gori DNA (500 fmol as circle) as prepared under “Experimental Procedures” in 50 μl of enzyme dilution buffer containing 10 mM magnesium acetate and 200 μM ATP by an incubation at 30°C for 5 min. Elongation reaction was initiated by the addition of each 48 pmol of dATP, dCTP, and dGTP and 18 pmol of [α-32P]dTMP (100 cpm/μmol) at 30°C for 5 min. To block reinitiation during polymerase cycling so that processivity could be measured, 20 μg of anti-β IgG was added to tubes (lanes 4, 5, 8, and 9) prior to dNTP addition. Anti-β IgG was omitted from lanes 2, 3, 6, and 7. Subsequent steps were performed as described in Fig. 8. Lanes 1 and 10 are controls in the absence of polymerases. As a control to demonstrate that anti-β IgG under these reaction conditions can rapidly inhibit cycling polymerase, tubes containing all components except polymerase (either α or αβθ), DnaX complex, and β either in the presence or absence of anti-β IgG (20 μg) were incubated at 30°C for 5 min, immediately added to a mix of polymerase, DnaX complex, and β and incubated for an additional 15 s and 1 min for αβ and αβθ-reconstituted holoenzymes, respectively. Radioactivity of [α-32P]dTMP-incorporated DNA was measured as described (McHenry and Crow, 1979), and anti-β IgG inhibited 90% of holoenzyme activity. Letters on the right side of the gel indicate the DNA fragments (a–j) generated by restriction enzyme digestion as described in Fig. 8.

**DISCUSSION**

Three subunits of pol III are tightly associated and can be isolated from wild-type cell lysates (McHenry and Crow, 1979). Since a cell contains only 10–20 molecules of pol III (Wu et al., 1984), purification of pol III from wild-type E. coli required a 30,000-fold purification and enormous quantities of cells (McHenry and Crow, 1979). We have constructed an artificial operon containing combinations of the three pol III genes (dnaE, dnaQ, and holE) which permitted overexpression of three pol III subunits from a single promoter and the assembly of complexes in vivo. From 180 g of pol III overexpressing cells, a complex β followed by incubations for sufficient time for replication of a full circle of M13Gori DNA (15 s for pol III and 1 min for α). The intensity of each fragment in the same lane was determined by a densitometric scan. Comparison of ratios of early fragments (a, b, or d) and late fragments (f or g) in the presence or absence of anti-β IgG showed that once holoenzyme is reconstituted with either α or αβθ using τ complex to initiate DNA synthesis, it processively replicates M13Gori DNA (until fragment g). However, the ratio of early fragments and one particular late fragment (h) of α-reconstituted holoenzyme using τ complex was 3–4-fold higher in the absence of anti-β IgG (lane 2) than in its presence (lane 4), indicating that only 25–30% of initiation complexes completed the synthesis of fragment h in the presence of anti-β IgG during the time course of the reaction. Perhaps the movement of holoenzyme is impeded by a strong hairpin in the bacteriophage M13 replication origin (van Wezenbeek et al., 1980) located in fragment h.

**In Vivo Assembly of Overproduced DNA Pol III**

**Fig. 8. Determination of the elongation rate of α, αβ, and αβθ in reconstituted holoenzyme.** A, scheme of elongation assay. The initiation complex on RNA-primed, SSB-coated M13Gori DNA was formed at 30°C for 5 min. The reaction was started at 22°C by the addition of dNTPs. The numbers on replicated double-stranded DNA indicate the distance of BbvI cleavage sites from the 3′-OH primer terminus. Fragments generated from the restriction enzyme digestion are given as a (900 bp), b (550 bp), c (660 bp), d (1,639 bp), e (661 bp), f (1,584 bp), g (261 bp), h (2,300 bp), i (688 bp), and j (256 bp). Details are described under “Experimental Procedures.” The arrow in the initiation complex indicates the direction of DNA synthesis. H, DNA polymerase III holoenzyme reconstituted using α complex, β, and either α, αβ, or αβθ. B, restriction gel analysis. The α-reconstituted holoenzyme (lanes 1–6), αβ-reconstituted holoenzyme (lanes 7–12), and αβθ-reconstituted holoenzyme (lanes 14–20) were incubated at 22°C for various times: 0 (lane 1), 1 (lane 2), 2 (lane 3), 5 (lane 4), 10 (lane 5), and 20 min (lane 6) for α-reconstituted holoenzyme; 0 (lanes 7 and 14), 10 (lanes 8 and 15), 20 (lanes 9 and 16), 30 s (lanes 10 and 17), 1 min (lanes 11 and 18), 2 min (lanes 12 and 19), and 5 min (lanes 13 and 20) for αβ and αβθ-reconstituted holoenzymes. The DNA fragments (a–j) were separated by 8% native polyacrylamide gel electrophoresis and stained with ethidium bromide.

**Fig. 9. Processivity of α-reconstituted holoenzyme.** Initiation complexes were formed by addition of either τ (lanes 1–5) or γ (lanes 6–10) complex (1.2 pmol as τG or γG, β) (565 fmol as dimer), and either 1 pmol of α (lanes 2, 4, 6, and 8) or αβθ (lanes 3, 5, 7, and 9) to primed M13Gori DNA (500 fmol as circle) as prepared under “Experimental Procedures” in 50 μl of enzyme dilution buffer containing 10 μl of enzyme dilution buffer containing 10 mM magnesium acetate and 200 μM ATP by an incubation at 30°C for 5 min. Elongation reaction was initiated by the addition of each 48 pmol of dATP, dCTP, and dGTP and 18 pmol of [α-32P]dTMP (100 cpm/μmol) at 30°C for 5 min. To block reinitiation during polymerase cycling so that processivity could be measured, 20 μg of anti-β IgG was added to tubes (lanes 4, 5, 8, and 9) prior to dNTP addition. Anti-β IgG was omitted from lanes 2, 3, 6, and 7. Subsequent steps were performed as described in Fig. 8. Lanes 1 and 10 are controls in the absence of polymerases. As a control to demonstrate that anti-β IgG under these reaction conditions can rapidly inhibit cycling polymerase, tubes containing all components except polymerase (either α or αβθ), DnaX complex, and β either in the presence or absence of anti-β IgG (20 μg) were incubated at 30°C for 5 min, immediately added to a mix of polymerase, DnaX complex, and β and incubated for an additional 15 s and 1 min for αβ and αβθ-reconstituted holoenzymes, respectively. Radioactivity of [α-32P]dTMP-incorporated DNA was measured as described (McHenry and Crow, 1979), and anti-β IgG inhibited 90% of holoenzyme activity. Letters on the right side of the gel indicate the DNA fragments (a–j) generated by restriction enzyme digestion as described in Fig. 8.
18 mg of pol III was purified to 99% homogeneity in two chromatographic steps. It is interesting that ε, normally insoluble when overproduced alone (Sheuermann and Echols, 1984), forms a defined 1:1 complex with α when coexpressed in vivo, resulting in a soluble complex. Excess ε is found in inclusion bodies.

Gap-filling polymerase assays were used to determine the contribution of ε or η to the kinetic properties of α. All three polymerase forms, α, αε, and αεθ, had identical affinities for dNTP, suggesting that ε and η are not involved in dNTP binding by α. The Kₐ₅₀ of α for DNA was about 2-fold higher than αεθ. The Kₑ₅₀ of α was 2-fold lower than that of αε and αεθ, indicating a modest contribution from ε, perhaps by stabilizing a more active conformation of α; η made no detectable contributions, consistent with other biochemical (Studwell-Vaughan and O’Donnell, 1990) and genetic (Slater et al., 1994) studies.

To complete the synthesis of 4.4-megabase pairs of the E. coli genome within 40 min, holoenzyme must synthesize DNA at a rate of about 1 kb per s. In vitro replication assays have shown that naturally purified as well as reconstituted holoenzyme synthesize DNA at a rate of about 500 nucleotides/s at 30 °C (Johanson and McHenry, 1981). Our gel analysis of restriction fragments indicated a DNA synthesis rate of the holoenzyme reconstituted with pol III, β, and η complex of about 130 nucleotides per s at 22 °C. The αε-reconstituted holoenzyme synthesized DNA at the same rate as αεθ-reconstituted holoenzyme, but the α-reconstituted holoenzyme elongated DNA at a rate 5-fold slower. Therefore, rapid DNA synthesis by holoenzyme is dependent on ε. By contrast, η appears to have no influence on DNA elongation at 22 °C. Although the α-reconstituted holoenzyme using η complex showed a 5-fold slower elongation rate than holoenzyme reconstituted using αε or αεθ, its processivity was very similar to that of the αεθ-reconstituted holoenzyme. When γ complex replaced η complex, the processivity of α-reconstituted holoenzyme decreased (Fig. 9). γ protects β from removal by γ complex (Kim et al. 1996a). Presumably, in the absence of γ, the slower moving α provides more time for β removal, resulting in a lower apparent processivity.

The function of η in DNA replication is not clear. Analysis of a null mutation in holoE has shown that η is dispensable for E. coli growth (Slater et al., 1994). Our kinetic and functional studies of three polymerases did not show any significant effect of η in DNA replication, although it slightly increased α’s binding to DNA substrates. The η subunit has also been shown to slightly stimulate the 3’→5’ proofreading exonuclease activity of the ε subunit at a mismatched base pair (Studwell-Vaughan and O’Donnell, 1993). Another possible role of η is communication with other replication proteins such as primase or helicase at the replication fork. One of two holoenzymes at the replication fork continuously recycles onto newly synthesized primer after completion of the synthesis of a preceding Okazaki fragment, which requires a signal between a new primer and subunits of holoenzyme (Wu et al., 1992). Clearly a η-DnaB interaction is required for replicase-primeosome coupling (Kim et al., 1996b) but that does not preclude additional primosome-holoenzyme interactions. Finally, η might somehow mediate the conformational change in pol III that is probably necessary in switching between the polymerase and exonuclease activity of pol III during replication. Firm conclusions about the function of η await further genetic, structural, and functional studies.

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