Constitution of the Twin Polymerase of DNA Polymerase III Holoenzyme*

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It is speculated that DNA polymerases which duplicate chromosomes are dimeric to provide concurrent replication of both leading and lagging strands. DNA polymerase III holoenzyme (holoenzyme), is the 10-subunit replicase of the Escherichia coli chromosome. A complex of the α (DNA polymerase) and ε (3'-5' exonuclease) subunits of the holoenzyme contains only one of each protein. Presumably, one of the eight other subunits functions to dimerize the α polymerase within the holoenzyme. Based on dimeric subassemblies of the holoenzyme, two subunits have been elected as possible agents of polymerase dimerization, one of which is the τ subunit (McHenry, C. S. (1982) J. Biol. Chem. 257, 2657-2663). Here, we have used pure α, ε, and τ subunits in binding studies to determine whether τ can dimerize the polymerase. We find τ binds directly to α. Whereas α is monomeric, τ is a dimer in its native state and thereby serves as an efficient scaffold to dimerize the polymerase. The ε subunit does not associate directly with τ but becomes dimerized in the αε complex by virtue of its interaction with α. We have analyzed the dimeric αε complex by different physical methods to increase the confidence that this complex truly contains a dimeric polymerase. The τ subunit is comprised of the NH2-terminal two-thirds of τ but does not bind to αε, identifying the COOH-terminal region of τ as essential to its polymerase dimerization function. The significance of these results with respect to the organization of subunits within the holoenzyme is discussed.

DNA polymerase III holoenzyme (holoenzyme)1 is the major replicative polymerase of the Escherichia coli chromosome (Kornberg, 1982). In common with replicases of phage T4, yeast, Drosophila, and mammals, the holoenzyme is comprised of a DNA polymerase subunit accompanied by multiple accessory proteins (Kornberg, 1982). The holoenzyme contains at least 10 different proteins in all (α, ε, θ, τ, γ, δ, δ', χ, Ψ, β) (Maki et al., 1988). One may presume by their very existence that each protein has a distinct function.2 Study of subassemblies and isolated subunits of the holoenzyme has led to assignment of function to at least half of the subunits so far. Thus, the heterotrimer core subassembly (αεθ) contains the polymerase and proofreading 3'-5' exonuclease activities (McHenry and Crow, 1979). Genetic overproduction and subsequent purification of individual subunits identified α as the polymerase (Maki and Kornberg, 1985) and ε as the 3'-5' exonuclease (Scheuermann and Echols, 1985). The core polymerase has only low catalytic efficiency (20 nucleotides/s, Maki and Kornberg, 1987) and processivity (10-15 nucleotides/binding event, Fay et al., 1981). In contrast, the holoenzyme hydrolyzes ATP to initiate remarkably rapid (500 nucleotides/s) and processive (>8 kilobases) DNA synthesis (Fay et al., 1981; Burgers and Kornberg, 1982). The accessory proteins which confer the ATP-activated speed and processivity onto the holoenzyme were identified by studying which subunits restored these hallmark features onto the core polymerase. Rapid and highly processive synthesis was found to be rooted in formation of a tightly bound preinitiation complex of accessory proteins on primed DNA (Wickner, 1976; Maki and Kornberg, 1988b; O'Donnell, 1987). In assembly of the preinitiation complex, the five protein γ complex (γδδ'χψ) hydrolyzes ATP to transfer the β subunit from solution to the primed template (Wickner, 1976; Maki and Kornberg, 1988b; Stukenberg et al., 1991). Once β is transferred to primed DNA by the γ complex, the β is a mobile "sliding clamp" on duplex DNA and also binds the core polymerase thereby tethering it to the DNA for highly processive synthesis (Stukenberg et al., 1991). Studies aimed at determining the minimal subunit requirements of the γ complex for its action in assembling the β clamp showed only the γ and δ subunits were essential to transfer β onto primed DNA (O'Donnell and Studwell, 1990). Studies to determine which subunits of core were needed for processive synthesis showed the β clamp could not provide α with highly processive synthesis but was able to confer processive synthesis onto a complex of α (Studwell and O'Donnell, 1990).

The studies summarized above have assigned functions to only five subunits of the holoenzyme (α, ε, γ, δ, β). Due to their limited availability or lack of obtaining them in pure form, little is known about the functions of the θ, δ', χ, and ψ subunits. Several properties have been assigned to the τ subunit although its full role in holoenzyme action is still not certain. The studies presented in this report focus on the ability of τ to dimerize two polymerase subunits.

A four-subunit subassembly of holoenzyme, called polIII'...
is a complex of $\tau$ with core (McHenry, 1982). The $\alpha$ and $\tau$ subunits of polIII' appeared to be equimolar based on their staining intensity in a polyacrylamide gel (McHenry, 1982). Further, hydrodynamic studies indicated a size of polIII' (410 kDa) consistent with two of each subunit (McHenry, 1982). Hydrodynamic studies of the core polymerase indicated that core (160 kDa) contained only one copy of $\alpha$ (McHenry, 1982). Hence, it was hypothesized that $\tau$ dimerized the core. Consistent with a dimeric polymerase within the holoenzyme, hydrodynamic measurements of the holoenzyme indicate a mass of 900 kDa, approximately twice that predicted from summation of the monomeric molecular mass of each of the subunits (Maki et al., 1988). Two polymerase subunits within the holoenzyme fits nicely with proposals that replicative polymerases act in pairs for smooth and simultaneous replication of both leading and lagging strands (Sinha et al., 1980; Kornberg, 1982).

Subsequent to characterization of polIII', the core polymerase was shown to be capable of dimerizing on its own indicating $\tau$ is not essential for polymerase dimerization (Maki et al., 1988). Since the $\alpha \tau$ complex showed no tendency to dimerize, the $\theta$ subunit was elected as the probable agent of core dimerization (Maki et al., 1988). Furthermore, reconstitution of the processive polymerase on primed DNA indicated a $\tau$ dimer bound only one core suggesting the subunits of polIII' are arranged such that one $\tau$ dimer is bound to only one side of a core dimer (Maki et al., 1988). This subunit arrangement of polIII' indicates that $\tau$ does not function to dimerize core by binding to two core molecules. It would appear more likely that the two cores within polIII' become dimerized by interaction between their $\delta$ subunits, and the method used to purify polIII' yielded the dimeric core with a $\tau$ dimer still bound to one of the core halves.

The gene which encodes the $\tau$ subunit also encodes the $\gamma$ subunit (Kodaira et al., 1983). The $\gamma$ subunit is comprised of the amino-terminal two-thirds of $\tau$ and is formed by an efficient translational frameshift event such that approximately equal amounts of $\tau$ and $\gamma$ are produced from the same dnaZ X gene (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). The relationship of $\gamma$ to $\tau$ suggests they may share some functions. In fact, the $\tau$ subunit can act with either $\delta$ or $\delta'$ to transfer $\beta'$ to primed DNA suggesting either $\delta$ or $\delta'$ complex may fulfill a similar role to $\gamma$ (O'Donnell and Studwell, 1990). $\tau$ differs from $\gamma$ inasmuch as $\tau$ is a DNA-dependent ATPase, whereas $\gamma$ only binds ATP utilizing the COOH-terminal region of $\gamma$ interacts with DNA (Lee and Walker, 1987; Tsuchihashi and Kornberg, 1989). That $\tau$ binds DNA but $\gamma$ does not has not been demonstrated (Discussion in Tsuchihashi and Kornberg, 1989). The ability of $\tau$ to bind both core and DNA likely forms a common basis underlying observations of others on the effect of $\tau$ on core polymerase activity. Thus, $\tau$ increases the processivity of core (Fay et al., 1982), increases the affinity of core for a preinitiation complex on primed DNA (Maki and Kornberg, 1988), and aids the reconstituted processive polymerase in replication past regions of secondary structure (Maki and Kornberg, 1988).

The relationship between $\gamma$ and $\tau$ implied these two subunits would have similar molecular interactions within the holoenzyme and led to proposals that a $\tau$ dimer was bound to one core, and a $\gamma$ dimer (of the $\gamma$ complex) was bound to the other half of the dimeric holoenzyme (Hawker and McHenry, 1987; Maki et al., 1988; McHenry, 1988). Hence, the related $\tau$ and $\gamma$ subunits were hypothesized as the basis of a structural asymmetry within the holoenzyme wherein a different set of accessory proteins were bound to the two halves of a core dimer. The $\gamma$ half of the holoenzyme, proposed to contain the $\gamma$ complex, core, and $\beta$, seemed suited to the lagging strand where the $\gamma$ complex is needed to repeatedly initiate replication of lagging strand fragments. The $\tau$ half of the holoenzyme, proposed to contain $\tau$, core, and $\beta$, appeared suited to the leading strand where the DNA-binding domain of $\tau$ could anchor the polymerase to DNA for extensive polymerization.

In this report we have used pure $\alpha$, $\epsilon$, $\tau$, and $\gamma$ subunits to define the interactions between them. We have utilized hydrodynamic measurements but also other techniques to increase the confidence in conclusions about dimeric complexes. The $\theta$ gene has only recently been identified and therefore $\theta$ was not available for these studies. Nevertheless, the results obtained with the four subunits studied here demonstrate that $\tau$ dimerizes $\alpha$ without any other subunits. Further, the $\gamma$ subunit showed no affinity for the polymerase. Implications of these results on the organization of subunits within the holoenzyme are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources were: unlabeled and labeled nucleotides, Pharmacia LKB Biotechnology Inc. and Du Pont-New England Nuclear, respectively. x174 ssDNA was prepared by banding the phage down and then up in two successive cesium chloride gradients as described (Ray, 1989). x174 ssDNA was uniquely primed with a synthetic DNA 30-mer (map position 2828–2794) as described (Studwell and O'Donnell, 1990). Protein standards for molecular mass studies were from Bio-Rad and Sigma. Proteins were purified to homogeneity as described: $\alpha$ (Maki and Kornberg, 1985), $\epsilon$ (Scheuermann and Echols, 1985), $\alpha$ (Studwell and O'Donnell, 1990), $\tau$ and $\gamma$ (Maki and Kornberg, 1988), $\beta$ (O'Donnell et al., 1986), $\gamma$ complex (Maki and Kornberg, 1988b), and SSB (Weiner et al., 1975). The concentration of $\beta$ was determined by absorbance using a molar absorption coefficient of 17,900 M$^{-1}$cm$^{-1}$ (Johanson et al., 1986). The concentration of $\gamma$ was determined by amino acid analysis. The concentrations of $\alpha$, $\epsilon$, SSB, $\gamma$ complex, and $\delta$ were determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976). The concentrations of $\alpha$ polymerase and $\tau$ subunit were determined from their absorbance at 280 nm and their tryptophan fluorescence (explained below). The molar extinction coefficient at 280 nm of a protein in its native state can be calculated from its gene sequence to within $\pm$5% (Gill and von Hipple, 1989). Extinction coefficients for $\alpha$, $\epsilon$, $\gamma$ (Table II) were calculated from their gene sequences (respectively, Tomaszewicz and McHenry, 1987; Maki et al., 1983; Flower and McHenry, 1986) using the equation, $\epsilon_{\text{nm}} = 12,600$ $\text{M}^{-1}$ $\text{cm}^{-1}$ (Johanson et al., 1986) where $n$ and $m$ are the number of tryptophan and tyrosine residues, respectively, in each subunit. The molar extinction coefficients of tryptophan and tyrosine were from Edelhoch (1967). Proteins were dialyzed to remove DTT prior to absorbance measurements. The concentration of the $\alpha$ polymerase was taken as the average of measurements by 1) absorbance at 280 nm using $\epsilon_{280} = 107,530$ $\text{M}^{-1}$ $\text{cm}^{-1}$ for a 1:1 complex of $\alpha$ and $\epsilon$, 2) by the tryptophan fluorescence intensity of the $\alpha$ polymerase (excitation at 280 nm, emission at 340 nm) in 6 M guanidine hydrochloride in 20 mM Tris-Cl (final pH 7.5) using N-\text{ethyl} tryptophan ethyl ester as standard and the knowledge of 9 tryptophan residues in a 1:1 complex of $\alpha$. Likewise, concentration of $\tau$ was the average of measurements by 1) absorbance at 280 nm (using $\epsilon_{280} = 45,560$ $\text{M}^{-1}$ $\text{cm}^{-1}$ (as monomer)) calculated from its gene sequence, and 2) measurement of its tryptophan fluorescence intensity (6 tryptophan residues) in 6 M guanidine hydrochloride. The protein concentration determined by absorbance and by fluorescence agreed within 20%.

**Buffers**—Buffer A is 25 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM EDTA, and 0.3 M NaCl. Buffer B is 20 mM Tris-Cl, pH 7.5, 8 mM
Constitution of a Twin Polymerase

MgCl₂, 5 mM DTT, 0.1 mM EDTA, 4% glycerol, and 40 µg/ml bovine serum albumin. Buffer C is 25 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM DTT, and 0.1 mM NaCl. Buffer D is 0.05% HPLC grade trifluoroacetic acid (Baker Chemical Co.) and 0.05% triethylamine (Baker) in HPLC grade water (Baker).

Specific Subunit Assays—Activity assays for specific subunits were performed by using them in combination with other subunits to reconstitute the characteristic processive polymerization of the holoenzyme (O'Donnell and Studwell, 1990). Two µl of column fraction was added to 72 ng of primed φX174 ssDNA, 0.88 µg of SSB, 0.5 mM ATP, 60 µM dCTP, 60 µM dGTP, 12 ng of β, 140 ng of α, 50 ng of ε, and 4 ng of γ complex in a final volume of 25 µl of Buffer B. Reactions were incubated for 6 min at 37 °C. Replication was initiated upon addition of dATP and [α-32P]dCTP (specific activity, 3,000-10,000 cpm/pmole) to final concentrations of 60 and 40 µM, respectively, and quenched after 15 s with 25 µl of 1% SDS, 40 mM EDTA. Incorporation of radiolabel was measured by absorption to DE81 paper (Whatman) as described by Rowen and Kornberg (1978). Only the reconstituted processive polymerase which assembles on the primed DNA in the 6-min preincubation gives rise to products in the short 15-s replication time (Maki and Kornberg, 1988b; O'Donnell and Studwell, 1990). Reconstitution of processive activity requires α, β, and either the γ complex, γ6 or γ6 (or γ6') (O'Donnell and Studwell, 1990). Therefore, assays of γ used 30 ng of δ in place of the γ complex, fractions containing δ were assayed using 30 ng of δ in place of the γ complex, and assays of α, α, and ε were performed by omitting the respective subunit(s) from the assay. Assays of α, α, and (αγδ), in Fig. 5 were performed by omitting α from the assay.

Gel Filtration—Proteins were incubated together for 1 h at 15°C in 200 µl of Buffer A then injected onto a 30-ml fast protein liquid chromatography Superose 6 column. Columns were developed with Buffer A and fractions of 180 µl were collected. Fractions were assayed for specific subunits as described above and by 12% SDS-polyacrylamide gels (100 µl of fraction) stained with Coomassie Brilliant Blue (Laemmli, 1970). The Stokes radius of each protein complex was determined upon comparison of its elution volume with that of proteins of known Stokes radius analyzed in a parallel column.

Glycerol Gradient Sedimentation—Proteins were incubated together for 1 h at 15°C in 300 µl of Buffer C containing 10% glycerol then layered onto 11.6 ml of 10-30% linear glycerol gradients in Buffer C. Gradients were centrifuged at 40,000 rpm in a Beckman SW Ti-41 rotor for 20 h at 4°C. Fractions of 180 µl were collected from the bottom of each tube. Fractions were assayed for specific subunits and were analyzed on 12% SDS-polyacrylamide gels (100 µl of fraction). The sedimentation coefficient of protein complexes was determined by comparison to proteins of known sedimentation coefficients in a parallel glycerol gradient.

HPLC—Reversed-phase HPLC analysis was performed on a Beckman SW Ti-41 rotor for 20 h at 4°C. Fractions of 180 µl were collected and assayed for activity as described above and by 12% SDS-polyacrylamide gel filtration (Fig. 1) and glycerol gradient analysis (Fig. 2). The gradient fractions were analyzed by SDS-PAGE (Fig. 2A, right panel) and activity assays (Fig. 2A, left panel). Recovery of polymerase activity from the gradient was 79% of the total activity loaded onto the gradient. The α polymerase was also analyzed by glycerox gradient analysis (Fig. 2). The gradient fractions were analyzed by SDS-PAGE (Fig. 2A, right panel) and activity assays (Fig. 2A, left panel). Recovery of polymerase activity from the gradient fractions was 79% of the total activity loaded onto the gradient. The α polymerase sedimented with a value of 6.9 S upon comparison with proteins of known S value (Fig. 2D). This value is close to the 7 S measurement of the core polymerase (obtained upon inspection of Fig. 6 of McHenry, 1982).
The Stokes radius and S value were combined in the equation of Siegel and Monty (1966) to calculate a mass of 145 kDa for the α polymerase, similar to 157.5 kDa predicted from the gene sequence of a 1:1 α complex (Table I).

The σ subunit has previously been analyzed by gel filtration (Stokes radius, 85 Å) and glycerol gradient sedimentation (6.8 S) (Tsuchihashi and Kornberg, 1989). It was noted that σ sedimented as a dimer in the glycerol gradient but behaved as an asymmetric shape or possibly a higher aggregation state in the gel filtration analysis. Analysis of the σ complex in the Siegel and Monty equation yielded a molecular mass of 206 kDa (Table I), a value more consistent with the predicted mass of a σ trimer (213 kDa) than a σ dimer (142 kDa). We will presume here the extended shape of σ overestimates its size and therefore σ is a dimer, although a trimeric state for σ cannot be rigorously excluded.

Mixture of σ with molar excesses of α and ε shows efficient formation of an ασ complex (i.e. all the σ is in complex with α and ε) which eluted from the gel filtration column much earlier (Fig. 1C) and sedimented in the glycerol gradient faster (Fig. 2C) than either σ or the α polymerase alone. The SDS-PAGE analysis (right panels) and reconstitution assays (left panels) showed all the σ co-migrated with the α polymerase as an ασ complex. The ασ complex cleanly resolved from the excess αε. Out of the total polymerase activity applied to the gel filtration column, 56% was recovered in the ασ peak and 25% was recovered in the ασ peak for a total recovery of 81%. Eighty-eight % of the r activity was recovered from the gel filtration column, and all of it was in the ασ peak. Of the polymerase activity loaded onto the glycerol gradient, 64% was present in the ασ fractions and 20% was localized in the αε fractions for a total recovery of 84%. All of the r activity loaded onto the glycerol gradient was recovered (~95%) and was located entirely within the ασ fractions. The fact that all the σ is found in association with αε even at these slight molar excesses of αε (over r) demonstrates that the constitution of σ with αε is highly efficient.

The Stokes radius (100 Å) and S value (12 S) of the ασ complex, although not identical to the reported values for polIII, are similar to the measured Stokes radius (85 Å) and S value (11.3 S) of polIII (McHenry, 1982). Combination of the Stokes radius and sedimentation coefficient of the ασ complex in the Siegel and Monty equation yielded a mass of 520 kDa, closer to 457 kDa calculated for a subunit stoichiometry of (ασ)₂ than 300 kDa calculated for an ασ₂ complex (Table I) or a 371 kDa ασ₂ complex or 684 kDa (ασ)₃ complex.

The Molar Ratio of Subunits in the ασ Complex—We next determined the molar ratio of subunits in the ασ complex to further distinguish between the ασ₂ or (ασ)₃ assignment.

### TABLE I

| Physical properties of α, τ, and the ασ complex |
|-----------------------------------------------|
| Stokes radius (Å) | 50 | 67 | 100 |
| Sedimentation coefficient | 6.9 | 7.0 | 12.0 |
| Partial specific volume (cm³/g)* | 0.736 | 0.742 | 0.738 |
| Native molecular mass (Da) | 148,000 | 206,000 | 520,000 |
| Molecular mass from gene sequences (Da) | 157,000 | 71,000 | 300,000 | 142,000 | 371,000 | 213,000 | 457,000 | 684,000 |
| Molecular weight and frictional coefficient | 1.42 | 1.70 | 1.87 |

*The partial specific volume was calculated by summation of the known partial specific volumes of the individual amino acids (Cohn and Edsall, 1943). The theoretical partial specific volumes of α and ε were 0.737 and 0.735 cm³/g, respectively.
Molar ratios of subunits in the αβ complex

Molar ratios of subunits in the αβ complex isolated by gel filtration were calculated from the densitometer tracing (Fig. 3C) upon dividing the observed peak area of a subunit by the peak area of that respective subunit in the 1:1:1 molar ratio standard (Fig. 3A). The values obtained for α, τ, and ε were normalized to the value for α. Molar ratios of subunits in the glycerol gradient purified αβ complex (Fig. 3D) were determined likewise. In the HPLC analysis, the molar ratio of subunits in the αβ complex was determined by monitoring their elution from the HPLC column at 220 nm and 280 nm (see “Experimental Procedures”). The peptide bond is the major chromophore at 220 nm, and thus the molar ratio of subunits was calculated by dividing the area under each peak (Fig. 4A) by the number of amino acids in the respective subunit and normalized relative to the value for α. The areas under the peaks of 280-nm absorbance were divided by their respective ε_{280} (see “Experimental Procedures”) and normalized relative to the value for α. The number of amino acids and the ε_{280} values for α, ε, and τ were determined from their respective gene sequences.

| SDS-PAGE densitometry | α  | ε  | τ  |
|-----------------------|----|----|----|
| HPLC 220-nm absorbance|    |    |    |
| Amino acid residues   | 1,160 | 243 | 643 |
| Molar ratio*          | 1.0 | 0.8 | 1.2 |
| HPLC 280-nm absorbance|    |    |    |
| Tyrosine              | 8   | 1  | 6  |
| Extinction coefficient | 95,440 | 12,090 | 45,660 |
| Molar ratio*          | 1.0 | 0.9 | 1.2 |

* Average of three determinations (±0.02 for ε, ±0.05 for τ).

The peptide bond absorbs strongly at 220 nm, and therefore the area under the peak of absorbance is directly proportional to the number of amino acids in the respective protein. The molar ratio, calculated by dividing the peak area by the number of amino acids, was 1:0.81:3 for α, ε, and τ, respectively (Table II). In a parallel analysis, elution of α, ε, and τ from the HPLC column was monitored at 280 nm where the amino acids tryptophan and tyrosine absorb (Fig. 4B). The molar extinction coefficients of α, ε, and τ were calculated from the number of tryptophan and tyrosine residues in each subunit predicted from their respective genes (Table II). A molar ratio of 1:0.91:2 for α, ε, and τ, respectively, was calculated by dividing the area under the peak of 280-nm absorbance by the extinction coefficient of the respective subunit (Table II). Whereas the molar ratio of α to τ in the αβ polymerase is one-to-one (Maki and Kornberg, 1987; Studwell and O'Donnell, 1990), the αβ complex appears on average 25% deficient in ε (Table II). Perhaps the interaction of τ with ε destabilizes the binding of ε to α.

The nearly equimolar ratio of subunits in the αβ complex combined with its large molecular weight indicates the (αβ)_2 stoichiometry rather than the αβ_2 stoichiometry. An αβ_2 complex is unlikely in light of the dimeric structure of τ and large size of the (αβ)_2 complex. Further evidence for the (αβ)_2 assignment is given below.

**Test of the dimeric Polymerase—Assembly of two polymerases on a τ dimer scaffold allows a testable prediction.** Namely, under limiting ατ polymerase (molar excess of τ), each τ dimer should obtain only one ατ molecule thus forming
FIG. 4. HPLC analysis of the molar ratio of subunits in the αε complex. Separation of the subunits of Superose 6 purified αε complex (60 µg) on a reverse-phase C4 HPLC column was performed as described under "Experimental Procedures." Elution of subunits was monitored at 220 nm (A) and 280 nm (B). The assignment of a peak with its respective subunit, shown above each peak, was determined upon collecting the peak, evaporating the sample to dryness, and analysis in a 12% SDS-polyacrylamide gel (not shown).

an αε7 complex as diagrammed in Fig. 5A. This prediction assumes the two molecules of αε bind independently (without high cooperativity) to the τ dimer. Indeed, constitution of αε with a 5-fold molar excess of τ dimer yielded the αε7 complex (Fig. 5B, triangles). The αε7 complex eluted from the gel filtration column later (Stokes radius of 91 Å) than the dimeric (αε)2 complex (Fig. 5B, circles) but earlier than either αε or τ alone (Fig. 1A and B), consistent with the predicted size of an αε7 complex (300 kDa). Visualization of the subunits in the SDS-polyacrylamide gel showed the αε7 complex did not fully resolve from the excess free τ subunit (data not shown) which precluded subunit molar ratio studies on the αε7 complex.

Subunit Contacts in the (αε)2 Complex—We used gel filtration to show the τ subunit binds the αε polymerase through contact with α (Fig. 6, C and D). Mixture of τ with a molar excess of α followed by gel filtration resulted in an (αε)2 complex which consumed all the τ subunit (Fig. 6C). The left panel in Fig. 6C shows all the τ activity coeluted with the α activity. The SDS-polyacrylamide gel analysis confirmed the coelution of α and τ (right panel, Fig. 6C). In this experiment, 83% of the τ activity was recovered from the column, and all of it was in the (αε)2 peak. Of the α activity loaded onto the column, 49% eluted with the (αε)2 complex and 32% was present as free α for a total recovery of 81%. The (αε)2 assignment is based on 1) the molar ratio 1:0.9 (α/τ) obtained by densitometry analysis (not shown) of the gel-filtered (αε)2 complex in the Coomassie Blue-stained polyacrylamide gel (Fig. 6C), and 2) the large Stokes radius (97 Å) of the (αε)2 complex determined from its elution volume (nearly the same as the (αε)2 complex) from the gel filtration column (Fig. 6C).

Incubation of a mixture of τ and ε did not yield a complex in the gel filtration analysis (Fig. 6D). The SDS-PAGE analysis showed ε did not comigrate with τ in the gel filtration fractions (Fig. 6D, right panel). Furthermore, ε activity did not comigrate with τ activity (Fig. 6D, left panel).

The γ subunit of the holoenzyme is derived from the same gene as τ by a translational frameshift event which occurs after synthesis of approximately two-thirds of τ (Tsuchihashi and Kornberg, 1990; Flower and McHenry, 1990; Blinkowa and Walker, 1990). The frameshift is followed within a few codons by a stop codon. Therefore, except for one amino acid near the COOH terminus, γ is comprised of the NH2-terminal 431 amino acids of τ. We examined the γ subunit for ability to bind αε (Fig. 6, A and B). Previous hydrodynamic measurements of γ showed it had a Stokes radius of 62 Å and S value of 6.6 (Tsuchihashi and Kornberg, 1989). Gel filtration of γ alone showed it eluted (Stokes radius, 63 Å) near the position of τ (Fig. 6A) indicating the γ subunit, like τ, has an extended structure. The γ subunit sediments (6.8 S) slightly slower than τ in a glycerol gradient consistent with the smaller size of a γ dimer (data not shown). The γ, αε and ε subunits were incubated together and then gel-filtered, but there was no evidence of complex formation between αε and γ (Fig. 6B). The γ subunit eluted in the same position whether αε was present or not (compare Fig. 6, A with B). Likewise, the elution volume of αε was unchanged by γ (compare Fig. 1A with Fig. 6B). Recoveries of γ and αε activity from the column were 80 and 77%, respectively. We have repeated the gel filtration analysis in lower salt (100 mM NaCl) both in the presence and absence of 0.5 mM ATP and 8 mM MgCl2 with
Constitution of a Twin Polymerase

We have reconstituted the twin polymerase of DNA polymerase III holoenzyme. The polIII' core, α, is a monomer and remains so when complexed with β, the 3'-5' exonuclease subunit. The dimeric τ subunit binds two α polymerase molecules to form an (ατ), complex with an observed mass of 520,000 daltons (predicted mass of 456,000 Da). The three subunits are equimolar as expected for a (ατ), complex. These results are consistent with the initial proposal by McHenry (1982) that τ dimerizes core within the polIII' complex (ατ),. The polIII' complex is naturally purified from E. coli, evidence for the existence of the dimeric polymerase inside the cell. The intact holoenzyme purified from E. coli contains the α, ε, and τ subunits in approximately equimolar ratios and is large enough to accommodate a dimer of all the subunits (Maki et al., 1988) suggesting the presence of the (ατ), dimer within the holoenzyme.

The polIII' core (α, ε, and τ subunits) is reported to dimerize when sufficiently concentrated (18 μM) (Maki et al., 1988). Since the αε polymerase showed no tendency to dimerize, it was suggested that the ε subunit was the agent that mediated dimerization of core (Maki et al., 1988). The β subunit, and possibly even others, may contribute to polymerase dimerization, but the present work shows the τ dimer can achieve this function at a relatively low concentration (Kd ≤ 17 nM) in the absence of ε. Based on the estimate of 20 molecules of the holoenzyme/cell (McHenry and Kornberg, 1981), the intracellular concentration of its subunits would be approximately 40 nM. Hence, the binding strength of τ to αε is strong enough to ensure formation of the (ατ), complex at the level of these subunits found in vivo. We would like to study the relative contribution of ε to polymerase dimerization by the reconstitution approach, but these studies must await overproduction of ε as it has never been purified free of α and τ, and its gene has only recently been discovered.

The simplest interpretation of results in this report suggests the τ subunits are symmetrically disposed about the two αε polymerases (Fig. 7). The τ dimer is presented in Fig. 7 as long and thin to reflect its extended structure predicted from its Stokes radius and sedimentation coefficient. The γ subunit contains the NH2-terminal two-thirds of the τ protein but does not bind αε indicating that αε interacts with the COOH-terminal portion of τ. The two α subunits are dimerized by virtue of one α binding each half of the τ dimer. The τ subunit interacts with αε mainly through contact with α, since τ forms

\[ K_d = \frac{[\alpha\tau][\tau]}{[\alpha\tau][\tau]} = \frac{[22 \times 10^{-7}M][22 \times 10^{-7}M]}{28 \times 10^{-7}M} = 17 \text{ nM}. \]

This value may be an upper limit if equilibrium was not reached in the 24 h incubation.
a complex with α but not with ε. The ε subunit is shown bound only to α since a complex between τ and ε was not observed.

It has been hypothesized that the accessory proteins of the E. coli DNA polymerase III holoenzyme are disposed asymmetrically about the two polymerase subunits to confer the separate properties anticipated in replication of the leading and lagging strands (Maki et al., 1988; McHenry, 1988). The close relationship between the τ and γ subunits was proposed as the basis for an asymmetric orientation of accessory subunits about the two core polymerases (i.e. τ on one core and γ on the other) (Maki et al., 1988; McHenry, 1988). Ability to reconstitute a complex containing a τ dimer bound to a single core supported the notion that a dimer of γ may bind the other core molecule within the holoenzyme (Maki et al., 1988). However, in light of the studies presented here, the previous observation of a τ dimer bound to one core is likely explained by the 10-fold molar excess of τ dimer relative to core monomer used in the previous study. This would lead to formation of a core-τγ complex, much like the αετγ complex formed here using a molar excess of τ over αε (Fig. 5). Another previous observation which indicated γ bound to core was that addition of τ to primed DNA coated with SSB in the presence of core, β, and γ complex decreased the amount of γ complex bound to the DNA by 2-fold, possibly explained by competition of τ and γ for core (Maki et al., 1988). We did not detect an interaction of γ with αε in these studies, although it is possible that the interaction of γ with αε was too weak to detect it (Kd ≈ 5 μM, the concentrations of αε and γ used in the Fig. 6 experiment). With such a low affinity of γ for αε relative to the strength of the τ-αε interaction, it seems γ would have a difficult time ever appropriating a polymerase from τ in assembly of an asymmetric dimer. Despite the results presented here it is still possible that τ and γ are positioned on opposite halves of a polymerase dimer. For example, perhaps γ interacts with β or develops a strong interaction with core when γ is within the γ complex or in the presence of β and primed DNA (γ does not bind αε in the presence of β in solution, data not shown). It is also possible that τ competes with another subunit of the γ complex for core, although the asymmetry achieved by such an interaction would not be based in the relationship of γ to τ.

The COOH-terminal one-third of τ (missing in γ), besides a role in binding α, is also essential for the interaction between τ and DNA (evidence outlined in the Introduction). It has been proposed that since τ strengthens the grip of the polymerase to DNA, the τ subunit would not be present on the lagging strand where the polymerase must loosen its grip on DNA and dissociate from a completed Okazaki fragment in order to cycle to a new RNA primer (Maki et al., 1988). A mechanism by which the lagging strand polymerase can have a tight grip on DNA yet still rapidly dissociate from the DNA after it has completely replicated the available template has been addressed in our previous studies. Thus, core rapidly dissociates from the β clamp only after completing processive replication of the DNA template, followed by (or concerted with) rapid reassociation of the core with a new preinitiation complex (β clamp) on a new primed template (O'Donnell, 1987; O'Donnell and Studwell, 1990; Studwell et al., 1990). Further, τ did not prevent the polymerase from cycling to new primed templates (endowed with a β clamp) in this experimental system (O'Donnell and Studwell, 1990).

The simplest arrangement of subunits to explain how a τ dimer binds two αε polymerases is that a τ dimer bridges two αε polymerase molecules (e.g. Fig. 7). It is reasonable to presume that δ is also present on each half of the polymerase dimer since δ is isolated in a tight complex with αε (i.e. core). Additionally, each core must interact with a sliding clamp β dimer in order to become tethered to DNA for processive synthesis (Stukenberg et al., 1991). There are approximately two β dimers within the holoenzyme consistent with a β dimer for each half of the polymerase dimer (Maki et al., 1988). This leaves the γ complex subassembly to impose a structural asymmetry onto the holoenzyme. The γ complex may be needed only once to initiate processive replication of the polymerase on the leading strand, but the γ complex is needed repeatedly on the lagging strand to initiate processive replication by the lagging strand polymerase on each new primer. To determine whether only one γ complex is present in the holoenzyme asymmetrically disposed relative to the two polymerases will require further studies.

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