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The DnaX-binding Subunits δ′ and ψ Are Bound to γ and Not τ in the DNA Polymerase III Holoenzyme*

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The DnaX complex subassembly of the DNA polymerase III holoenzyme is comprised of the DnaX proteins τ and γ and the auxiliary subunits δ, δ′, χ, and ψ, which together load the β processivity factor onto primed DNA in an ATP-dependent reaction. δ′ and ψ bind directly to DnaX whereas δ and χ bind to δ′ and ψ, respectively (Onrust, R., Finkelstein, J., Naktinis, V., Turner, J., Fang, L., and O'Donnell, M. (1995) *J. Biol. Chem.* 270, 13348–13357). Until now, it has been unclear which DnaX protein, τ or γ, in holoenzyme binds the auxiliary subunits δ, δ′, χ,and ψ. Treatment of purified holoenzyme with the homobifunctional cross-linker bis(sulfosuccinimidyl)suberate produces covalently cross-linked γ-δ′ and γ-ψ complexes identified by Western blot analysis. Immunodetection of cross-linked species with anti-δ′ and anti-ψ antibodies revealed that no τ-δ′ or τ-ψ cross-links had formed, suggesting that the δ′ and ψ subunits reside only on γ within holoenzyme.

The DNA polymerase III holoenzyme consists of 10 different protein subunits (1, 2) and is the major replicative polymerase of *Escherichia coli,* responsible for synthesizing the entire bacterial chromosome. Like other replicases from eukaryotes and prokaryotes, the holoenzyme can be resolved into three primary functional units, a polymerase core, a sliding clamp processivity factor, and a clamp assembly apparatus. The core polymerase consists of the α subunit, which contains the DNA polymerase activity, ε, which provides a 3′-5′ exonuclease proofreading activity, and the θ subunit, which has no apparent function identified to date (3–7). In *E. coli*, the β subunit plays the role of the processivity factor (8, 9). $\tau_2\gamma_2\delta\delta'\chi\psi$ form a clamp loader complex, which loads β on primed templates in an ATP-dependent reaction (10–12).

The τ and γ subunits are both products of the *dna*X gene (13, 14); they comprise the ATPase that drives β loading on a primed template and replication complex assembly (10, 15–17). The γ subunit is a truncated version of τ arising from a −1 ribosomal frameshift (18–21). The carboxyl-terminal extension of τ, absent from γ, is responsible for dimerization of Pol III (12, 22, 23) and binding to the DnaB helicase, effectively coupling all the replicative activities of the fork into one complex (24–26).

δ′ and ψ bind directly to DnaX whereas δ and χ bind to δ′ and ψ, respectively (27). The DnaX complex[1] auxiliary subunits δ and δ′, which bind tightly to each other, play an important role in clamp assembly (12, 22, 23, 25, 28–31). χ and ψ perform an ancillary, nonessential role in simple single-stranded assays (27, 30, 31). The presence of χ and ψ makes the holoenzyme resistant to glutamate concentrations up to 800 mM and dramatically increases the affinity of DnaX for δ and δ′, dropping the K_d to a point where they saturate DnaX at physiological concentrations (31). χ-ψ binds to SSB in the presence or absence of other subunits of the DnaX complex, and the interaction of SSB with χ-ψ is responsible for the previously observed salt resistance conferred upon the holoenzyme by χ-ψ (32, 33).

In vitro, both γ and τ subunits can be assembled into functional DnaX complexes (τ complex and γ complex)($\tau_4\delta_1\delta'_1\chi_1\psi_1$ or $\gamma_4\delta_1\delta'_1\chi_1\psi_1$) (12, 27, 34). Both of the homomeric DnaX complexes are capable of loading the β clamp onto DNA in an ATP-dependent manner.

Holoenzyme contains both γ and τ (35), yet it has not been demonstrated which DnaX subunit binds the auxiliary subunits and sets the β_2 processivity factor on DNA. In this report, our focus is on the relative subunit arrangement within the clamp loader in the holoenzyme. We have employed the homobifunctional cross-linker BS[3] (bis(sulfosuccinimidyl)suberate) to determine, within authentic holoenzyme, which DnaX subunit binds the bridging auxiliary subunits δ′ and ψ.

EXPERIMENTAL PROCEDURES

Proteins, Antibodies, and Reagents—Pol III HE protein subunits were purified as follows: core (7), β (36), τ and γ (37), δ and δ′ by an unpublished procedure,[2] and χψ (31). The DNA polymerase III holoenzyme was purified according to Ref. 38. The fraction III holoenzyme sample used in Fig. 4 was purified as previously reported (38) with the following changes. The fraction III holoenzyme was placed in buffer H (50 mM HEPES (pH 7.2), 20% glycerol, 100 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol) by gel filtration on a NAP25 desalting column (Amersham Pharmacia Biotech) equilibrated in buffer H. The specific activity of holoenzyme before and after the NAP25 column was unchanged at 1.5×10^5 units/mg with a protein and activity recovery >85%. Monoclonal antibodies directed against the DnaX complex subunits were produced in collaboration with the University of Colorado Health Sciences Center Tissue Culture and Monoclonal Antibody Facility. Monoclonal antibodies used were from anti-γ cell line 527G1, anti-δ′ cell line 1538F2, anti-ψ cell line 1026G12, and anti-τ cell line 123-28. Antibodies were purified from 500 ml of hybridoma supernatant by 50% ammonium sulfate fractionation, resuspended in (50 ml) and dialyzed against phosphate-buffered saline (80 mM Na_2HPO_4, 20 mM NaH_2PO_4 (pH 7.5), 100 mM NaCl), and used in a 1:1000 dilution. Benchmark prestained protein ladder (Lot No. MCJ401) was from Life Technologies, Inc. BS[3] was purchased from Pierce.

Protein Determinations—Protein concentration determination for individual protein subunits was determined by extinction coefficient whereas protein complexes were determined using the Pierce Coomassie Plus assay according to the manufacturer's specifications. Bovine

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[1] The abbreviations used are: DnaX complex, a complex containing either or both products of the *dna*X gene (γ or τ) with associated δ, δ′, χ, and ψ; holoenzyme, *E. coli* DNA polymerase III holoenzyme; τ complex, a complex containing τ, δ, δ′, χ, and ψ; γ complex, a complex containing γ, δ, δ′, χ, and ψ; BS[3], (bis(sulfosuccinimidyl)suberate); FrIII, purification Fraction III from Ref. 38; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid ; SSB, single-stranded DNA-binding protein.

[2] M. Song, H. G. Dallmann, M. W. Olson, P. Pham, R. Schaaper, and C. S. McHenry, manuscript in preparation.

serum albumin (fat-free, Sigma) was used as an assay standard.

SDS-Polyacrylamide Electrophoresis and Immunodetection—Proteins were loaded onto a 5–17.5% gradient SDS-polyacrylamide gel (0.075 × 18 × 16 cm) and separated at 65 V overnight. The separated proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membrane at 500 mA for 6 h and blocked in MTBS (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% non-fat milk) overnight at 4 °C. Membranes were immunoblotted with DnaX complex subunit-specific antibodies (1:1000 dilution in MTBS). Immunostaining was visualized using a biotinylated secondary anti-mouse antibody (1:1000 dilution in MTBS) followed by horseradish peroxidase-conjugated streptavidin (1:1000 dilution in MTBS) and developed with the enhanced chemiluminescent (ECL) method (Amersham Pharmacia Biotech). Membranes were washed in TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) following incubations with the primary antibody, secondary antibody, and the horseradish peroxidase-conjugated streptavidin (1 × 15 min and 2 × 5 min).

Preparation of the DnaX Complex and Subassemblies—The τ and γ complexes were reconstituted and purified (12, 27) by incubating 10 nmol of DnaX with 15 nmol each of δ, δ′, and the χψ complex. Complexes were allowed to form at room temperature for 15 min, after which they were applied to a Mono Q (Amersham Pharmacia Biotech) FPLC column equilibrated in buffer M (25 mM HEPES (pH 7.4), 5% glycerol, and 50 mM NaCl). The column was developed using a 15-ml linear gradient from 100 to 400 mM NaCl in buffer M at a flow rate of 0.5 ml/min collecting 0.5-ml fractions. The τ and γ complexes eluted at 280 mM NaCl equivalent.

The DnaXχψ and DnaXδδ′ complexes were prepared as follows. 8 μM DnaX (γ_4 or τ_4) was incubated with 12 μM either χψ or δδ′ for 10 min at room temperature. 250 μl of the mixture was applied to a 24-ml Superose 12 FPLC column (Amersham Pharmacia Biotech) equilibrated in buffer S (50 mM MOPS (pH 7.4), 5% glycerol, 50 mM KCl, and 10 mM $MgCl_2$). The column was developed in buffer S at a flow rate of 0.2 ml/min while collecting 0.5-ml fractions. 20 μl from each fraction was subjected to 5–17.5% SDS-PAGE analysis and stained with Coomassie Brilliant Blue G-250 to determine the elution position of the DnaX subcomplexes. The concentration of the subcomplexes in pooled fractions was >1 μM.

BS^3 Chemical Cross-linking—BS^3 stock solutions were prepared fresh before every reaction by dissolving BS^3 in buffer B (50 mM HEPES (pH 7.4), 5% glycerol, and 50 mM NaCl). An aliquot of the BS^3 stock solution was added to the protein complex immediately and reacted at room temperature for 40 min. Reactions were quenched either by the addition of SDS-PAGE sample buffer or by the addition of 500 mM Tris-HCl, pH 6.8. DnaX complex and subcomplexes were cross-linked with 10 μM BS^3, purified holoenzyme with 500 μM BS^3, and fraction III holoenzyme with 600 μM BS^3 unless stated otherwise.

RESULTS

BS^3 Cross-linking of the DnaX Complex—BS^3 cross-links primary amine groups and is useful in the detection of associated protein subunits (39–43). We utilized BS^3 as an analytical tool in conjunction with monoclonal antibodies directed against DnaX complex protein subunits in examining the protein subunit arrangement within the DnaX complex (Fig. 1, *A–D*). We used the γ complex, a homomeric version of the DnaX complex, to optimize experimental conditions for the cross-linking reaction. Incubation of γ complex with BS^3 followed by immunoblotting with anti-γ antibody led to the appearance of additional immunoreactive bands, indicating that cross-linking had occurred between γ and protein subunits within the complex. γ was found in cross-linked protein complexes of 61 and 85 kDa, as well as in several species in the 90–187-kDa range. The degree of this cross-linking was a function of both the concentration of BS^3 (Fig. 2*A*) and the time of incubation with BS^3 (Fig. 2*B*). Quantitation of the appearance of the 61- and 85-kDa bands over time demonstrates that the reaction is still proceeding at 40 min (data not shown). At 40 min in the presence of 10 μM BS^3, the majority of the γ remains un-cross-linked as evidenced by the strong signal at 50 kDa. Additionally, the amounts of cross-linked complexes that were formed did not decrease with decreasing protein concentration demonstrating specifically that the 61- and 85-kDa bands arose from intra- and not intermolecular interactions (Fig. 2*C*).

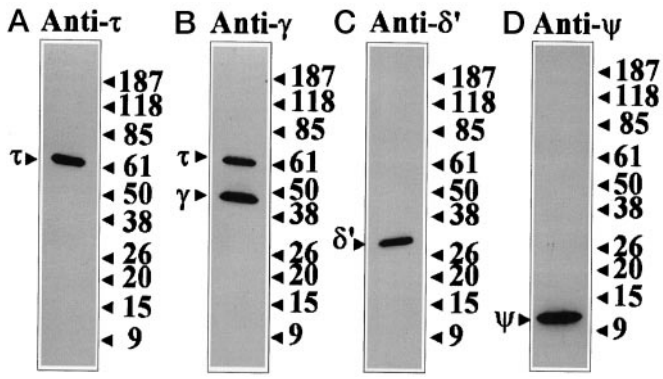

FIG. 1. **Specificity of DnaX complex subunit monoclonal antibodies.** Holoenzyme protein subunit standards were subjected to SDS-PAGE and Western blot analysis as described under "Experimental Procedures." *A–D* represent a single lane containing 10 pmol of each holoenzyme protein subunit blotted with a single monoclonal antibody directed against DnaX complex subunits (anti-τ, γ, δ′, ψ). The *arrows* designate the electrophoretic position of prestained protein markers.

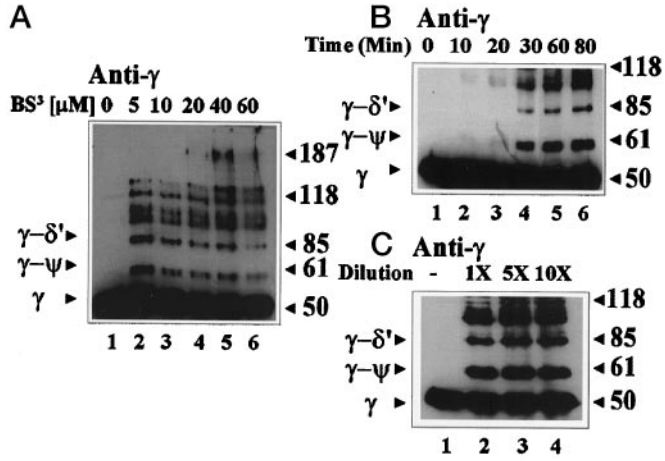

FIG. 2. **BS^3 cross-links auxiliary subunits to DnaX within the same complex.** *A*, γ complex (5 μg each lane) was cross-linked at room temperature for 40 min with increasing BS^3 concentrations, quenched, subjected to 5–17.5% SDS-PAGE, and immunoblotted with an anti-γ monoclonal antibody. *Lanes 1–6* correspond to BS^3 concentrations of 0, 5, 10, 20, 40, and 60 μM, respectively. *B*, γ complex (35 μg) was incubated at room temperature in the absence (*lane 1*) and presence of 10 μM BS^3 (*lanes 2–6*). 30-μl aliquots were removed from the cross-linking reaction and quenched at the indicated time points, subjected to 5–17.5% SDS-PAGE, and immunoblotted with an anti-γ monoclonal antibody. *C*, three concentrations of γ complex (9 μg total each lane) were cross-linked with 10 μM BS^3. *Lanes 1–4* represent cross-linking reactions containing 958 nM (no BS^3), 958 nM (1× dilution), 192 nM (5× dilution), and 96 nM (10× dilution) γ complex, respectively. After 40 min at room temperature the reaction products were quenched, trichloroacetic acid-precipitated, resuspended in SDS-PAGE sample buffer, and analyzed by immunoblot using an anti-γ monoclonal antibody. *A–C* immunoblots were developed with the ECL detection method.

BS^3 Cross-links DnaX with δ′ and ψ—Cross-linked γ complex produced bands that are consistent with binary covalent complexes of γ-ψ and γ-δ′ that have calculated molecular masses of 63 kDa and 85 kDa, respectively (Fig. 2). To investigate this possibility and identify the cross-link partner to γ in the 61- and 85-kDa bands, we cross-linked complexes containing various auxiliary subunit combinations. We cross-linked complexes containing no auxiliary subunits (γ), χψ only (γχψ), δδ′ only (γδδ′), and both χψ and δδ′ together (γ complex). The resultant complexes were immunoblotted with an anti-γ (Fig. 3*A*), anti-δ′ (Fig. 3*B*), and an anti-ψ antibody (Fig. 3*C*). The 85-kDa band appeared only in complexes containing δ′. The presence of δ′ in this band is confirmed when the experiment is immunoblotted with an anti-δ′ antibody (compare *lanes 1* and

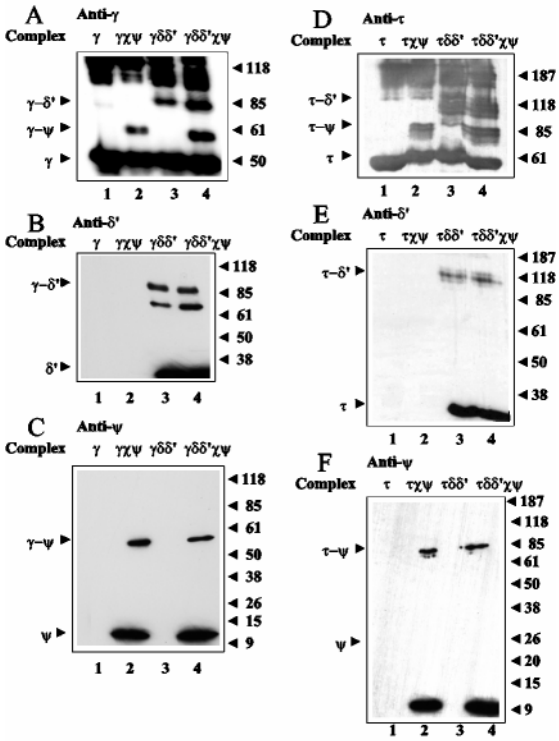

FIG. 3. **BS³ cross-links DnaX with δδ′ and χψ.** 30 pmol of γ (6 μg), γχψ (6 μg), γδδ′ (8 μg), and γδδ′χψ (10 μg) were each cross-linked with 10 μM BS³ for 40 min at room temperature and subjected to SDS-PAGE and immunoblotting in separate experiments with an anti-γ (*A*), anti-δ′ (*B*), and anti-ψ (*C*) monoclonal antibody. 30 pmol of τ (9 μg), τχψ (10 μg), τδδ′ (11 μg), and τδδ′χψ (12 μg) were each cross-linked with 10 μM BS³ for 40 min at room temperature and subjected to SDS-PAGE and immunoblotting in separate experiments with an anti-τ (*D*), anti-δ′ (*E*), and anti-ψ (*F*) monoclonal antibody.

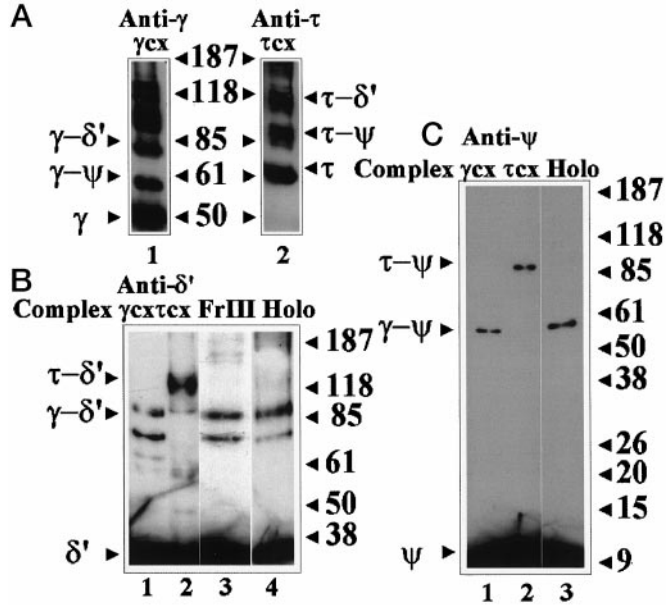

FIG. 4. **The auxiliary bridging subunits δ′ and ψ are located on γ and not τ in holoenzyme.** *A*, γ complex (γcx) (5 μg) and τ complex (τcx) (6 μg) were cross-linked with 10 μM BS³ for 40 min at room temperature, subjected to SDS-PAGE, and immunoblotted with either an anti-γ monoclonal antibody (*lane 1*) or an anti-τ monoclonal antibody (*lane 2*). γ complex (4 μg each lane), τ complex (5.2 μg each lane), FrIII (150 μg), and holoenzyme (10 μg each lane) were cross-linked with BS³ as described under "Experimental Procedures," immunoblotted, and developed with the ECL chemiluminescent method. *B*, BS³ cross-linked complexes blotted with an anti-δ′ monoclonal antibody. *C*, BS³ cross-linked complexes blotted with an anti-ψ monoclonal antibody.

2 with *3* and *4* in *A* and *B*). An additional band involving δ′ is evident migrating near 73 kDa. This band is not evident in the anti-γ blot (Fig. 3*A*, *lanes 3* and *4*) suggesting that γ is either not present in the 73-kDa band or is present as a degradation product lacking the anti-γ antibody epitope. This band is apparent in a complex containing only γ, δ, and δ′, eliminating the possibility that χ and ψ are present in this cross-link. The presence of ψ in the 61-kDa band is confirmed by blotting the cross-linked complexes with an anti-ψ antibody (Fig. 3*C*). Additionally, the 61-kDa band is evident only in complexes containing ψ (compare *lanes 2* and *4* with *1* and *3*). These results demonstrate that the 61- and 85-kDa bands contain covalently cross-linked complexes of γ-ψ and γ-δ′, respectively.

To determine whether τ cross-links to δ′ and ψ when present in DnaX complexes, we repeated the BS³ cross-linking experiments using complexes containing τ as the DnaX gene product. We observe that cross-linking of the τ complex and its subassemblies produce bands containing τ-δ′ and τ-ψ, which migrate at 118 kDa and 85 kDa, respectively. As in the case for the γ complex, the presence of τ, δ′, and ψ in the cross-link bands is confirmed by their immunodetection with anti-τ (Fig. 3*D*), anti-δ′ (Fig. 3*E*), and anti-ψ (Fig. 3*F*) antibodies.

We have demonstrated that BS³ cross-linking of the γ complex and τ complex results in DnaX-δ′ and DnaX-ψ covalent complexes (Fig. 4*A*). That these auxiliary subunits cross-link to both γ and τ in homomeric DnaX complexes is not surprising as the binding interface (and possibly the chemically reactive lysine) for the auxiliary subunits is within Domain III of DnaX, a region common to both γ and τ.[3]

[3] D. Gao and C. S. McHenry, manuscript in preparation.

The Bridging DnaX Complex Subunits δ′ and ψ Bind to γ within Holoenzyme—Unlike the *in vitro* reconstituted homomeric DnaX complexes, the DnaX complex within holoenzyme contains both γ and τ. Thus we expected that BS³ cross-linking of holoenzyme would produce cross-linked complexes of δ′ and ψ with either or both DnaX gene products. We cross-linked γ complex, τ complex, and holoenzyme with BS³ and analyzed the reactions by immunoblotting with antibodies to both δ′ and ψ. We included as a control (in the δ′ immunoblot) a cross-linking reaction involving holoenzyme from an initial purification state (FrIII) during the enzyme's purification procedure (38). The cross-linking results from Fraction III should mirror the results obtained from the fully purified holoenzyme demonstrating that no protein complex subunit rearrangement has occurred later in the purification process. In the δ′ immunoblot (Fig. 4*B*), comparison of the cross-linked holoenzyme lanes (*3* and *4*) with cross-linked γ complex (*lane 1*) and τ complex (*lane 2*) reveals that cross-linking of holoenzyme produces cross-link bands identical to those observed in the γ complex lane. A band involving δ′ running at 85 kDa is clearly evident in addition to the uncharacterized 73-kDa band seen previously (Fig. 3*B*, *lanes 3* and *4*). No τ-δ′ cross-link, which would migrate above 118 kDa (compare *lane 2* with *3* and *4*), is observed in either holoenzyme lane demonstrating that δ′ resides exclusively on the γ subunit in holoenzyme. The FrIII result does in fact mirror the one observed in the purified holoenzyme lane, confirming the stability of the subunit arrangement during the purification process.

We repeated the cross-linking experiment with γ complex, τ complex, and purified holoenzyme and immunoblotted with an anti-ψ monoclonal antibody (Fig. 4*C*). BS³ cross-linking of holoenzyme (*lane 3*) produces a single band approximately 61 kDa in size identical to one seen in the cross-linked γ complex lane.

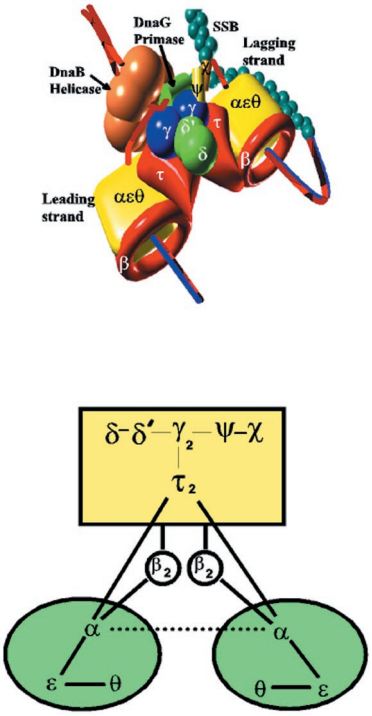

FIG. 5. **Model of holoenzyme structure.** This model reflects the results demonstrated in this report with the auxiliary subunits $\delta\delta'\chi\psi$ localized on the γ subunits.

No 88 kDa τ-ψ cross-link is observed demonstrating that ψ resides exclusively on the γ subunit in holoenzyme.

DISCUSSION

We have developed an analytical method for probing the protein subunit arrangement within the DnaX complex in holoenzyme. BS^3 covalently cross-links δ' and ψ to either DnaX gene product when present together in homomeric DnaX complexes. We exploited this tool to determine which DnaX protein in the holoenzyme's clamp loader binds δ' and ψ. The architecture of the holoenzyme shows that it has one set of δ' and ψ subunits per pair of polymerase cores, which we have now demonstrated reside on γ (Fig. 5).

Our findings in this report are consistent with previous evidence that τ and γ have differential interactions with replication proteins. From wild-type cells, γ can be isolated in a complex with δ, δ',χ, and ψ (10), whereas τ has only been isolated as a stable complex with Pol III or by itself (22). Additionally, the ATPase activity associated with β-loading onto primed DNA templates has been attributed to γ and not τ within a reconstituted Pol III* (44). This is consistent with our present report localizing the clamp loading apparatus to the γ subunit within authentic holoenzyme. Additionally, the placement of ψ on γ would suggest that the interaction of holoenzyme with SSB occurs through γ (Fig. 5). That γ binds the bridging auxiliary subunits provides support for the notion that γ is primarily involved in the clamp loading process. Although its role in replication has not been fully characterized, τ appears to function as an organizing protein localizing the clamp loader, SSB-binding, and DnaB helicase activities to the dimeric replicase at the replication fork.

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