

The DnaX-binding Subunits d^* and c Are Bound to g and Not t 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 t and g and the auxiliary subunits d , d^* , x , and c , which together load the b processivity factor onto primed DNA in an ATP-dependent reaction. d^* and c bind directly to DnaX whereas d and x bind to d^* and c , 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, t or g , in holoenzyme binds the auxiliary subunits d , d^* , x , and c . Treatment of purified holoenzyme with the homobifunctional cross-linker bis(sulfosuccinimidyl)suberate produces covalently cross-linked g - d^* and g - c complexes identified by Western blot analysis. Immunodetection of cross-linked species with anti- d^* and anti- c antibodies revealed that no t - d^* or t - c cross-links had formed, suggesting that the d^* and c subunits reside only on g 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 bac-

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

SDS-Polyacrylamide Electrophoresis and Immunodetection
Proteins were loaded onto a 5% gradient SDS-polyacrylamide gel (0.075 x 18 x 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 (15 min and 2 x 5 min).

Preparation of the DnaX Complex and Subassemblies
The α and β complexes were reconstituted and purified (12, 27) by incubating 10 nmol of DnaX with 15 nmol each of δ , ϵ , and the γ 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 Mr vis the

complexes
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2 with 3 and 4 in A and B). An additional band involving $d9$ is evident migrating near 73 kDa. This band is not evident in the anti- g blot (Fig. 3A, lanes 3 and 4) suggesting that g is either not present in the 73-kDa band or is present as a degradation product lacking the anti- g antibody epitope. This band is apparent in a complex containing only g , d , and $d9$, eliminating the possibility that x and c are present in this cross-link. The presence of c in the 61-kDa band is confirmed by blotting the cross-linked complexes with an anti- c antibody (Fig. 3C). Additionally, the 61-kDa band is evident only in complexes containing c (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 g - c and g - $d9$, respectively.

To determine whether t cross-links to $d9$ and c when present in DnaX complexes, we repeated the BS³ cross-linking experiments using complexes containing t as the DnaX gene product. We observe that cross-linking of the

that *g* is primarily involved in the clamp loading process. Although its role in replication has not been fully characterized, *t* 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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No 88 kDa *t*-*c* cross-link is observed demonstrating that *c* resides exclusively on the *g* subunit in holoenzyme.

DISCUSSION

We have developed an analytical method for probing the protein subunit arrangement within the DnaX complex in holoenzyme. BS³ covalently cross-links *d* and *c* 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 *d* and *c*. The architecture of the holoenzyme shows that it has one set of *d* and *c* subunits per pair of polymerase cores, which we have now demonstrated reside on *g* (Fig. 5).

Our findings in this report are consistent with previous evidence that *t* and *g* have differential interactions with replication proteins. From wild-type cells, *g* can be isolated in a complex with *d*, *d*_X, and *c* (10), whereas *t* has only been isolated as a stable complex with Pol III or by itself (22). Additionally, the ATPase activity associated with *b*-loading onto primed DNA templates has been attributed to *g* and not *t* within a reconstituted Pol III* (44). This is consistent with our present report localizing the clamp loading apparatus to the *g* subunit within authentic holoenzyme. Additionally, the placement of *c* on *g* would suggest that the interaction of holoenzyme with SSB occurs through *g* (Fig. 5). That *g* binds the bridging auxiliary subunits provides support for the notion

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