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# *Escherichia coli* DnaX product, the $\tau$ subunit of DNA polymerase III, is a multifunctional protein with single-stranded DNA-dependent ATPase activity

(DNA replication/*dnaZX* gene)

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**ABSTRACT** **The *dnaZX* gene of *Escherichia coli* directs the synthesis of two proteins, DnaZ and DnaX. These products are confirmed as the $\gamma$ and $\tau$ subunits of DNA polymerase III because antibody to a synthetic peptide present in both the DnaZ and DnaX proteins reacts also with the $\gamma$ and $\tau$ subunits of holoenzyme. To characterize biochemically the $\tau$ subunit, for which there has been no activity assay, the *dnaZX* gene was fused to the $\beta$-galactosidase gene to encode a fusion product in which the 20 C-terminal amino acids of the DnaX protein ($\tau$) were replaced by $\beta$-galactosidase lacking only 7 N-terminal amino acids. The 185-kDa fusion protein, which retained $\beta$-galactosidase activity, was overproduced to the level of about 5% of the soluble cellular protein by placing the gene fusion under control of the *tac* promoter and Shine–Dalgarno sequence. The fusion protein was isolated in one step by affinity chromatography on *p*-aminobenzyl 1-thio-$\beta$-D-galactopyranoside-agarose. The purified fusion protein also had ATPase (and dATPase) activity that was dependent on single-stranded DNA. This activity copurified with the $\beta$-galactosidase activity not only through the affinity column but also through a subsequent gel filtration. We conclude that the DnaX protein function involves binding to single-stranded DNA and hydrolysis of ATP or dATP, in addition to binding to other DNA polymerase III holoenzyme components, increasing the processivity of the core enzyme, and serving as a substrate for the production of the $\gamma$ subunit.**

*Escherichia coli* DNA polymerase III is a multisubunit complex composed of a core plus several auxiliary proteins (1–3). The core ($\alpha$, $\varepsilon$, and $\theta$ subunits) polymerizes DNA *in vitro* in the absence of auxiliary proteins, but the holoenzyme has greatly increased processivity (4). Moreover, the auxiliary factors are required for replication *in vivo* (e.g., ref. 5). Core components $\alpha$ and $\varepsilon$ are encoded by the *dnaE* and *dnaQ* genes, respectively (6, 7).

Auxiliary proteins assigned to the holoenzyme include $\beta$ (*dnaN*), $\gamma$, $\delta$, and $\tau$ (1, 2, 8). The $\gamma$ and $\tau$ proteins correspond to the DnaZ and DnaX proteins, respectively. These two proteins of $M_r$s 56,500 and 71,114, respectively, are the products of one gene called *dnaZX* and arise from one reading frame (9–12), possibly by proteolytic cleavage of the larger DnaX protein to generate the smaller DnaZ protein. The nucleotide sequence of the *dnaZX* gene contains a consensus ATP binding site (11), and the $\tau$ subunit has been shown to bind ATP (13). We report here the overproduction and purification of a biochemically active fusion protein consisting of the DnaX protein with its C-terminal 20 amino acids replaced by $\beta$-galactosidase lacking 7 N-terminal amino acids and the demonstration that this fusion protein has single-stranded DNA-dependent ATPase activity.

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Germino *et al.* (14) have used affinity chromatography to purify a bifunctional fusion protein consisting of the initiator of plasmid R6K replication fused near its C-terminal end to $\beta$-galactosidase.

## METHODS

**Bacterial Strains and Plasmids.** Strain RB791, a *lacI*$^Q$ L8 derivative of strain W3110 (15), was obtained from Nina Irwin (Harvard University). Strain M182, Δ(*lacIPOZYA*)-X74, *galK*, *galU*, *rpsL* (16), was obtained from Richard Meyer (University of Texas). pMC1403 (17) and pACYC184 (18) were obtained from Richard Meyer. ptac12 and placI$^Q$ (19) were obtained from Nina Irwin.

**Materials.** Restriction enzymes, T4 DNA ligase, BAL-31 nuclease, and DNA polymerase I Klenow fragment were obtained from New England Biolabs or Bethesda Research Laboratories and used as recommended. Peroxidase-labeled, affinity-purified, goat anti-rabbit IgG (H+L) and other immunoblotting reagents were from Kirkegaard and Perry Laboratories. *p*-Aminobenzyl 1-thio-$\beta$-D-galactopyranoside-agarose was from Sigma. Sephacryl S-200 was from Pharmacia.

**Enzyme Assays.** $\beta$-Galactosidase was assayed according to the method of Miller (20). The ATPase assay of Meyer *et al.* (21) was used, except that the source of single-stranded DNA was phage $\phi$X174 rather than phage G4. Double-stranded DNA was M13, replicative form II.

**Electrophoresis.** Samples were boiled for 5 min before analysis on 8% or 10% NaDodSO$_4$/PAGE gels. The gels were stained with Coomassie blue or the proteins were transferred to nitrocellulose for immunoblot analysis.

**Immunoblot.** Proteins separated on 8% NaDodSO$_4$/PAGE were transferred to 0.45-$\mu$m pore size nitrocellulose (22). After transfer, the portion of the nitrocellulose that contained the molecular weight markers was stained with KI/chloramine-T/starch (23). The remaining portion of the nitrocellulose was treated with 10% (wt/vol) nonfat dry milk to block unoccupied protein binding sites (24), exposed to antiserum prepared in rabbits to a synthetic peptide corresponding to amino acids 420–440 of both the DnaZ and DnaX proteins (S.-H.L., J.R.W., P. Kanda, and R. Kennedy, unpublished data), and incubated with peroxidase-labeled goat anti-rabbit IgG.

**Isolation of DnaX-$\beta$-Galactosidase Fusion Protein.** Cells of strain M182 containing plasmids pSL641 and pIQ were induced with 1 mM isopropyl $\beta$-D-thiogalactoside (IPTG) for 3.5 hr. They were harvested and lysed by the procedures of Huebscher and Kornberg (25), and the fusion protein was isolated by affinity chromatography. The procedure of

Abbreviations: SD, Shine–Dalgarno; IPTG, isopropyl $\beta$-D-thiogalactoside.

Germino *et al.* (14) was modified by substituting the *p*-aminobenzyl derivative for *p*-aminophenyl β-D-thiogalactosidyl succinyldiaminohexyl-Sepharose, and buffer A contained 5 mM dithiothreitol rather than 10 mM 2-mercaptoethanol.

## RESULTS

**Construction of a *dnaZX-lacZ* Fusion Under Control of the *tac* Promoter.** To facilitate overproduction and isolation of the DnaX protein, a *dnaZX-lacZ* gene fusion was constructed and then placed under control of the *tac* promoter and *lac* Shine–Dalgarno (SD) sequence. This fusion was constructed so as to retain (possibly) both DnaX and β-galactosidase activities by replacing 20 of the C-terminal triplets of *dnaZX* by the structural gene for β-galactosidase lacking the N-terminal 7 codons. After this fusion protein was determined to retain β-galactosidase activity in a preliminary experiment, the *dnaZX* promoter and SD sequence were removed by BAL-31 nuclease digestion so that the *tac* promoter and *lac* SD could be inserted. The ligation mixture was transformed into a recipient strain containing the $lacI^Q$ allele and plated in the presence of IPTG and 5-chloro-4-bromo-3-indolyl β-D-galactoside. Dark blue colonies (10% of the transformants) were tested for β-galactosidase activity after induction by IPTG. One strain, which synthesized 30,000-fold more β-galactosidase than a control strain in which the *dnaZX-lacZ* fusion was under control of the *dnaZX* promoter, yielded plasmid pSL641 (Fig. 1).

**Isolation of the DnaX-β-Galactosidase Fusion Protein.** A culture of cells containing pSL641 and pIQ (and with the chromosomal *lac* region deleted) was induced for 3.5 hr by 1 mM IPTG. The cells were harvested, lysed, and fractionated by precipitation with 40% saturated ammonium sulfate to generate fraction II as described by Huebscher and Kornberg (25). The DnaX-β-galactosidase fusion protein was visible after $NaDodSO_4$/PAGE as a 185-kDa band that contained ≈5% of the total fraction II protein. Affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside-agarose recovered 90% of the β-galactosidase activity, which was at least 90% pure, as judged by $NaDodSO_4$/PAGE (Fig. 2).

**ATPase Activity of the DnaX-β-Galactosidase Fusion Protein.** Yin *et al.* (11) reported that the deduced amino acid sequence of the DnaX protein contains a region that closely resembles the consensus ATP binding site A originally reported by Walker *et al.* (26). In addition, Biswas and Kornberg (13) reported that DNA polymerase III subunits τ and γ bind ATP. The purified DnaX-β-galactosidase protein was, therefore, assayed for ATPase and dATPase activities. The fractions from the affinity column that contained β-galactosidase activity also possessed ATPase activity in approximately constant ratio (Fig. 2*B*), suggesting that the fusion protein is also an ATPase. Moreover, antibody to the DnaX and DnaZ protein (see below) inhibited the ATPase activity of the purified fusion protein (S.-H.L. and J.R.W., unpublished data).

To determine if the ATPase and β-galactosidase activities were present in one protein, fractions 42 to 46 from the affinity column were pooled and subjected to gel filtration. Again, both activities copurified (Fig. 3). The lack of perfect correspondence between the two activities in this figure is likely more apparent than real because the low level of ATPase activity made accurate assays difficult. The fusion protein had both activities, suggesting that the DnaX protein is an ATPase. Removal of the C-terminal 20 amino acids during creation of the fusion protein did not inactivate the ATPase activity.

**The DnaX Protein ATPase Activity Is Single-Stranded DNA Dependent.** The purified DnaX-β-galactosidase fusion protein was assayed for DNA-dependent ATPase activity. The activity was stimulated over 10-fold by, and is likely to be dependent on, single-stranded DNA. Double-stranded DNA was essentially inactive. dATP is a more potent effector than ATP (Table 1).

**The DnaX Protein Is the τ Subunit of DNA Polymerase III.** It has been suggested that the DnaX protein corresponds to the τ subunit of holoenzyme because the two migrated

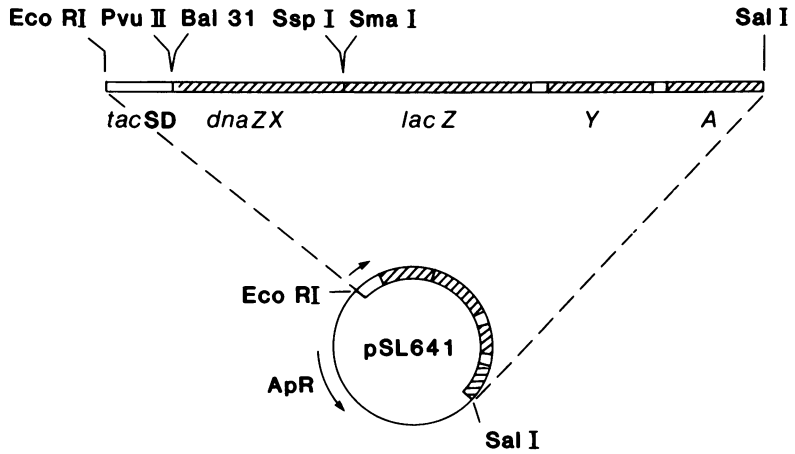


FIG. 1. Structure of pSL641 on which the *tac* promoter and SD control the expression of a *dnaZX-lacZ* fusion. pSL641 was constructed in two steps. First, a fragment containing an *Eco*RI end, the *dnaZX* promoter, SD sequence, and the N-terminal 623 *dnaZX* codons terminating in a blunt end (*Ssp* I end) (11) was prepared. pMC1403 (17), which carries ampicillin resistance and *lacZYA*, was cut with *Eco*RI and *Sma* I; *Sma* I also generated a blunt end and removed seven N-terminal codons of *lacZ*. Cloning the *Eco*RI–*Ssp* I fragment of *dnaZX* into the *Eco*RI–*Sma* I-treated pMC1403 resulted in formation of pSL581, which contains the *dnaZX* promoter and the *dnaZX* structural gene fused in phase with the *lacZ* cistron. Second, the *dnaZX* promoter and SD of pSL581 were replaced by the *tac* promoter and SD to generate pSL641. pSL581 was opened by *Eco*RI, and the *dnaZX* promoter and SD removed by limited digestion with BAL-31 nuclease. The Klenow fragment of DNA polymerase I was used to ensure the ends were blunt. A 270-base-pair *Eco*RI–*Pvu* II fragment containing the *tac* promoter and SD sequence was prepared from ptac12 (19) and ligated into pSL581 from which the *dnaZX* promoter had been removed. The hybrid plasmid that produced the highest level of β-galactosidase after induction was pSL641. pSL641 was maintained in host strain M182, which contains a deletion of *lacIPOZYA* (16) into which plasmid pIQ was introduced. pIQ is a $lacI^Q$-containing derivative of pACYC184, which is compatible with ColE1 replicons such as pSL641. pIQ was constructed by cloning a 1.1-kilobase-pair *Eco*RI fragment containing $lacI^Q$ from plasmid $placI^Q$ (19) into pACYC184 cut with *Eco*RI. The 5.1-kilobase-pair pIQ retains the tetracycline resistance marker of pACYC184. The bars indicate chromosomal DNA; the cross-hatched area is open reading frame.

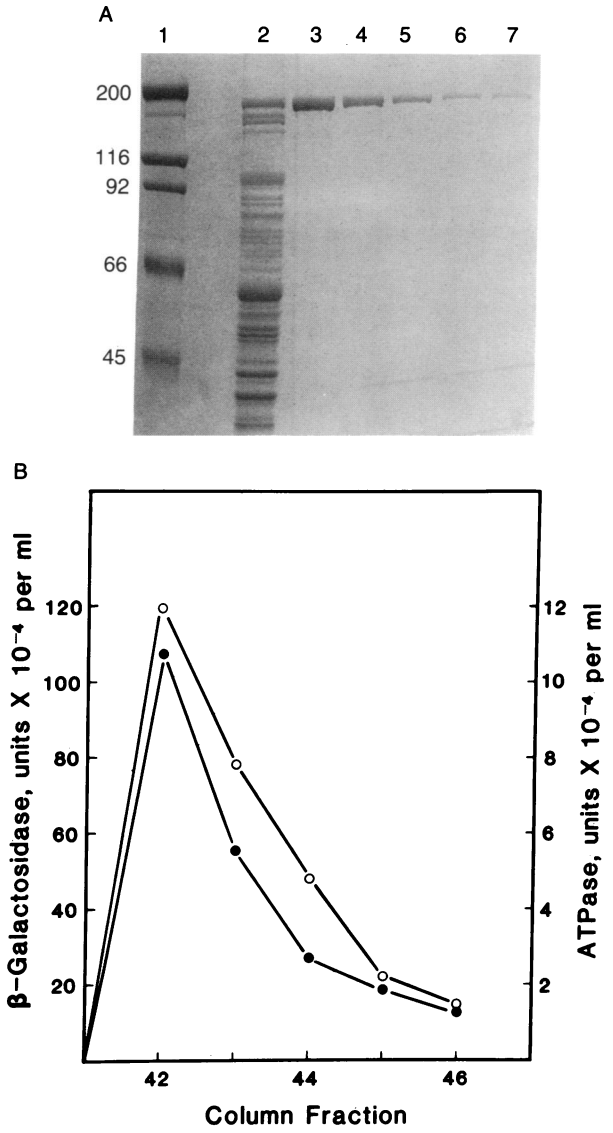


FIG. 2. Purification and activities of a DnaX-β-galactosidase fusion protein. (*A*) NaDodSO$_4$/PAGE and Coomassie blue staining of molecular size standards in kDa (lane 1), fraction II (lane 2), and column fractions 42–46 (lanes 3–7) after affinity chromatography on *p*-aminobenzyl 1-thio-β-D-galactopyranoside-agarose. Six liters of strain M182 containing pSL641 and pIQ was induced for 3.5 hr with 1 mM IPTG, harvested, lysed, and ammonium sulfate-fractionated to generate fraction II (25). Fraction II (5 ml) containing 130 mg of protein was applied to a 10-ml affinity column (14), which was washed with 10 column volumes of buffer A plus 0.1% Triton X-100 and 5 volumes of buffer A and then eluted with 0.1 M sodium borate buffer (pH 10.0). The fractions (4.1 ml) that contained β-galactosidase were immediately precipitated with ammonium sulfate, dissolved in 300 μl of buffer B, dialyzed against buffer B, and assayed for β-galactosidase and for ATPase. (*B*) β-Galactosidase and ATPase activities of the DnaX-β-galactosidase fusion protein. Column fractions 42–46, described in *A*, were assayed for β-galactosidase (●) and ATPase (○) activities.

similarly in electrophoresis (9, 10). That possibility was confirmed by the demonstration that antibody prepared against a synthetic peptide corresponding to amino acids 420–440, present in both the DnaZ and DnaX proteins (S.-H.L., J.R.W., P. Kanda, and R. Kennedy, unpublished data), reacted also with the τ and γ subunits. Cellular extracts and a purified preparation of DNA polymerase III holoenzyme were subjected to NaDodSO$_4$/PAGE, the separated proteins were transferred to nitrocellulose, and the blot was

Table 1. ATPase activity of the DnaX-β-galactosidase fusion protein is single-stranded DNA dependent

| Mononucleotide | DNA | ATPase or dATPase activity* |
|---|---|---|
| ATP | Single-stranded φX174 | 109 |
| ATP | Double-stranded M13 RFII | 15 |
| ATP | None | 8.5 |
| dATP | Single-stranded φX174 | 152 |

A 3-μl aliquot of column fraction 43 in Fig. 2 was assayed. RF, replicative form.
*Units per μl of column fraction 43 in Fig. 2.

probed with the synthetic peptide antibody. Fig. 4 demonstrates that the antibody reacted with the DnaX-β-galactosidase fusion protein (lane 2), with wild-type DnaX protein (lane 3), with DnaZ protein (lanes 2 and 3), and with the τ and γ subunits of holoenzyme (lane 4). This confirms that DnaX and Z proteins are the τ and γ subunits, respectively.

The antibody to the DnaX and Z proteins was elicited with a synthetic peptide corresponding to amino acids 420–440 that did not involve the ATP binding site (amino acids 49–59) (11). Specificity of the antibody for a region other than the consensus ATP binding site was supported by the demonstration that the antibody did not react with a protein known to contain a consensus ATP-binding sequence, helicase II (27) (S.-H.L. and J.R.W., unpublished data). Thus, the antibody reaction with purified τ and γ was specific and was probably not the result of binding to the consensus ATP binding peptide present in τ and γ.

The reaction of antibody with the τ subunit was not due to the presence of contaminating ATPase IV that is known to be present in holoenzyme preparations and to migrate similarly to the τ subunit (21). First, purified ATPase IV migrated as a 78-kDa band whereas the τ subunit (71.1 kDa) migrated at a position corresponding to 75 kDa. Second, purified ATPase IV did not bind to the DnaX protein antibody (S.-H.L. and J.R.W., unpublished data).

## DISCUSSION

Fusion of the *dnaZX* gene to the β-galactosidase gene has permitted the isolation of a fusion protein with both DnaX and β-galactosidase activities. The ATPase specific activity was 6–10 mol of ADP per mol of fusion protein per min, which is very low. By comparison, the specific activity of the *E. coli* RecA protein, one of the least active ATPases (28), is 25–27 mol of ADP per mol of protein per min (29, 30). Moreover, the ATPase activity of the DnaX-β-galactosidase fusion protein is very labile—its activity decreased by a factor of ≈10 during gel filtration (Figs. 2 and 3), and similar losses of activity were observed during other purification procedures. Whether the low activity and lability reflect intrinsic properties of the protein or result from fusion to β-galactosidase (by virtue of either deletion of the C-terminal 20 amino acids or physical linkage to the β-galactosidase) is not clear.

The *dnaZX* gene directs the synthesis of two proteins. DnaX is a $M_r$ 71,114 product, and DnaZ corresponds to the N-terminal 498 amino acids of the 643-amino acid DnaX (S.-H.L., J.R.W., P. Kanda, and R. Kennedy, unpublished data). Both these proteins are present in DNA polymerase III holoenzyme preparations. The DnaZ protein is thought to be the γ subunit of DNA polymerase III for several convincing reasons. First, Wickner and Hurwitz (31) purified the wild-type DnaZ protein by assaying for complementation of the *dnaZX*(Ts)2016 mutant, and this mutant extract was later complemented by purified γ (25). Second, the cloned *dnaZX* region stimulated overproduction of DnaZ and γ activities

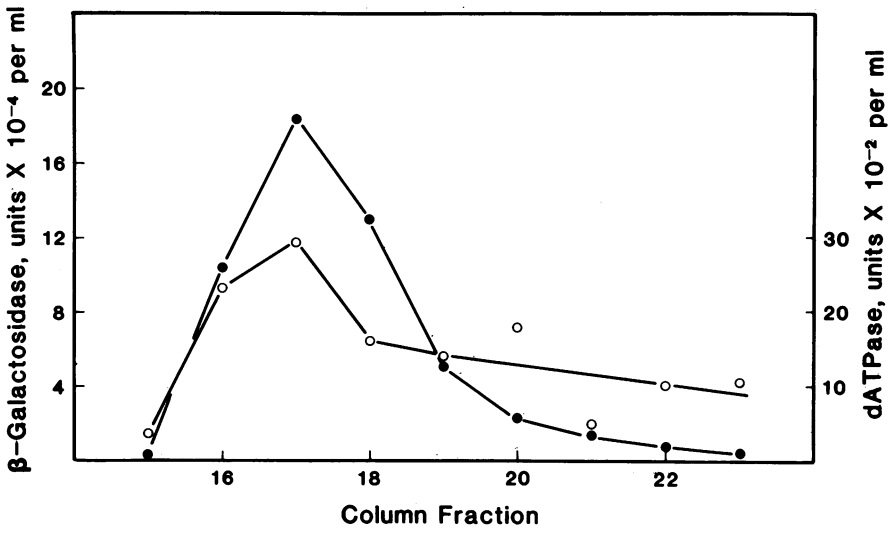


FIG. 3. ATPase and β-galactosidase activities of the DnaX-β-galactosidase fusion protein after gel filtration. Column fractions 42–46 of Fig. 2 were pooled, precipitated, redissolved in 1.4 ml of buffer B, and loaded onto 15.2 ml of Sephacryl S-200 in a column 0.9 × 26.8 cm equilibrated with buffer B (25). Filtration was carried out at a flow rate of 3 ml/hr. Fractions were assayed for β-galactosidase (●) and dATPase activity (○).

(25, 32, 33). Third, antibody raised to a synthetic peptide corresponding to residues 420–440 in both the DnaZ and DnaX proteins reacted with the γ subunit of DNA polymerase III holoenzyme (Fig. 4). Thus, it is clear that the DnaZ protein is present in holoenzyme preparations and probably corresponds to γ, although the question of how purified γ subunit was able to complement the *dnaZX*(Ts)2016 mutant

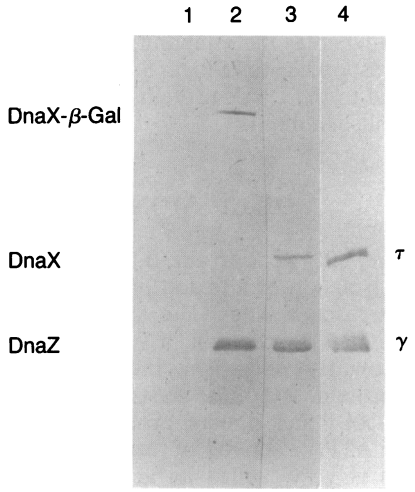


FIG. 4. Antibody to a synthetic peptide corresponding to 21 amino acids present in both the DnaX and DnaZ proteins reacts with the τ and γ subunits of DNA polymerase III. Proteins were subjected to NaDodSO$_4$/PAGE, transferred to a nitrocellulose sheet, and probed with antiserum. Lanes: 1, fraction II from strain M182; 2, fraction II from strain M182 carrying pSL641 (*dnaZX-lacZ* fusion under control of the *tac* promoter); 3, fraction II from strain RB791 containing pSL689, on which the intact *dnaZX* gene is under control of the *tac* promoter; 4, DNA polymerase III holoenzyme. Lanes 1–3 contained 2.5 μg of protein; lane 4 contained 2 μg. pSL689 was constructed in two steps. First, a 2.2-kilobase *Sma* I–*Pvu* II fragment containing the *dnaZX* promoter and structural gene was cloned downstream of the *tac* promoter in ptac12 to generate ptacZX, which contains both the *tac* promoter and the *dnaZX* promoter. Second, an *Aat* II fragment containing the *tac* promoter fused directly to the *dnaZX* structural gene was cut from pSL641 (Fig. 1) (*Aat* II conveniently cuts upstream of the *tac* promoter and also within the *dnaZX* structural gene near the N-terminal end) and used to replace an *Aat* II fragment of ptacZX containing both the *tac* and *dnaZX* promoter. The resulting plasmid was designated pSL689.

(25), which should be deficient in both DnaZ and DnaX activities, remains unanswered.

It was suggested that the DnaX protein is the τ subunit of DNA polymerase III because it migrated during two-dimensional NaDodSO$_4$/PAGE and isoelectric focusing with the τ subunit (10). However, there has been no functional test for DnaX or τ *in vitro* because the mutant thought to be defective in DnaX activity was not temperature-sensitive in the *in vitro* single-stranded to replicative form DNA assay (J.R.W., unpublished data). Although it has not been possible to demonstrate that purified τ complements a mutant extract or that a mutant extract contained temperature-sensitive τ, the antibody binding to both DnaX and τ (Fig. 4) is strong evidence of their identity.

The $M_r$ 56,500 DnaZ (γ) protein is likely to be generated from the $M_r$ 71,114 DnaX protein by proteolysis, rather than by translation of a truncated message, because the processing can be demonstrated *in vitro* (S.-H.L., J.R.W., P. Kanda, and R. Kennedy, unpublished data). This cleavage could be the result of a separate protease or of autodigestion.

The DnaX (τ) protein is multifunctional. First, it associates with DNA polymerase III core to form DNA polymerase III′ (α, ε, θ, and τ subunits) (34) and must, therefore, bind to one or more of the α, ε, and θ subunits. Second, τ increases the processivity of core because DNA polymerase III′ is intermediate in processivity between core and holoenzyme using randomly primed phage fd DNA as template (35). Third, the τ subunit is thought to serve as a substrate, the cleavage of

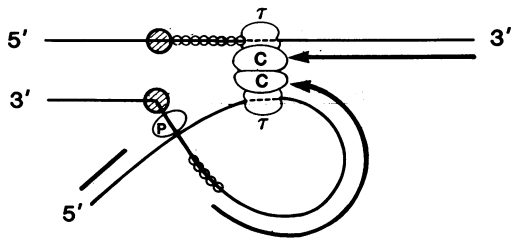


FIG. 5. The trombone model of polymerization (36) in which a DNA polymerase III holoenzyme dimer polymerizes leading and lagging strands simultaneously. τ binds the single-stranded template and other holoenzyme components and translocates the holoenzyme along the template using ATP or dATP hydrolysis. C, core or holoenzyme; P, primosome; the cross-hatched circles, helicases; the small circles, single-stranded binding protein.

which generates the $\gamma$ protein. Fourth, $\tau$ binds and hydrolyzes ATP and dATP (Figs. 2 and 3). Fifth, $\tau$ also binds single-stranded DNA because the ATPase activity is dependent on single-stranded DNA as an effector (Table 1).

These observations suggest at least four possible models for $\tau$ action. First, $\tau$ could enhance the binding of DNA polymerase III core or holoenzyme to single-stranded templates. Alternatively, or in addition, it could serve to translocate the core or holoenzyme along the template strand. In this model (Fig. 5), $\tau$ binds the template strand and another subunit(s) of DNA polymerase III and uses hydrolysis of ATP (or dATP) for translocation of the holoenzyme. It is also possible that $\tau$ is a helicase or functions to stimulate a helicase involved in replication. Finally, the ATP binding and hydrolysis might be essential to cleavage of $\tau$ to form the obligatory $\gamma$ subunit.

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