This report identifies the dnaX homolog from *Thermus thermophilus*. Replicases from bacteria to humans contain subunits that are homologous to one another. These homologs are subunits of a clamp loading apparatus that loads sliding clamps onto DNA, which in turn acts as mobile tethers for the replication machinery. In *Escherichia coli*, two of these subunits (γ and τ) are encoded by one gene (dnaX) in nearly equal amounts by way of an efficient translational frameshift. The γ and τ subunits form the central touchpoint that holds together two DNA polymerases with one clamp loading apparatus to form the *E. coli* chromosomal replicase, DNA polymerase III holoenzyme. The *E. coli* holoenzyme is an efficient replication machine that simultaneously replicates both strands of duplex DNA. The *T. thermophilus* dnaX homolog also contains a frameshift signature and produces both τ and γ-like proteins. Recombinant *T. thermophilus* τ and γ-like proteins, expressed in *E. coli*, have an oligomeric state similar to that of their *E. coli* counterparts and display ATPase activity that is stimulated by DNA. These results imply that *T. thermophilus* utilizes a DNA polymerase III holoenzyme replication machinery similar to that of *E. coli*.

Chromosomal replicases are composed of several subunits in all organisms (1). In keeping with the need to replicate long chromosomes, these multicomponent replicases are rapid and highly processive. Cellular chromosomal replicases derive their processivity from a protein subunit that is shaped like a ring and completely encircles DNA (2, 3). This “sliding clamp” protein acts as a mobile tether for the polymerase machinery (4). The sliding clamp does not assemble onto DNA by itself but requires a complex of several proteins, called a “clamp loader,” that couples ATP hydrolysis to the assembly of sliding clamps onto DNA (5). The three components of the *Escherichia coli* replicase, DNA polymerase III holoenzyme, are the three protein DNA polymerase III core (αεθδ), the β subunit DNA sliding clamp, and the five protein γ complex clamp loader (γδδ′ χψθ) (for review, see Ref. 6). In eukaryotes, from yeast to humans, the three components are the DNA polymerase δ, the PCNA sliding clamp, and the five protein Replication Factor C (RFC) clamp loader (for review, see Ref. 3).

The crystal structure of the circular sliding clamps from a variety of organisms have been determined (7–9), as have the crystal structures of the δ′ subunit (10) and εψ complex of the clamp loader apparatus from *E. coli*. With the aim to crystallize larger complexes, we have started to isolate the genes encoding the replicase subunits from a thermophile as heat stable proteins are often more amenable to crystal structure analysis.

As a beginning to identify and characterize the replicase of a thermophile, we started by looking for a homolog to the *E. coli* dnaX gene that encodes two protein subunits (γ and τ) of the DNA polymerase III holoenzyme through an efficient translational frameshifting mechanism (11–13). The dnaX gene has another homolog, holB, which encodes yet another subunit (δ′) of the holoenzyme (14, 15). The amino acid sequences of the δ′ and τ/γ proteins are conserved in replicases of other bacteria and in eukaryotes (14, 16–19).

The organism we chose to study was the extreme thermophile, *Thermus thermophilus*. Indeed, we identified a *T. thermophilus* homolog of dnaX. The gene encodes a full-length protein of 529 amino acids. The N-terminal third of the sequence shares over 50% identity to dnaX genes as divergent as *E. coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive). The *T. thermophilus* dnaX homolog contains a DNA sequence that provides a translational frameshift signal for production of two proteins from the same gene. Such frameshifting within dnaX has previously only been documented in *E. coli*.

The two protein subunits produced from the *E. coli* dnaX gene form the central core of the replicase, and they organize its three components into a holoenzyme particle (20). The τ subunit dimer holds two core polymerases together (21, 22), and it also forms a mixed heterotetramer (τ2γδ′) upon association with the γ subunit of the clamp loader, thereby bringing one clamp loading assembly (γ complex) into the large holoenzyme (18 polypeptides, 830 kDa) (20). This organized holoenzyme assembly acts at a replication fork to simultaneously replicate both strands of duplex DNA in a highly coordinated fashion (23–25).

The γ protein is encoded by the same gene that encodes the τ protein (dnaX). γ is essentially the N-terminal 2/3 of τ; it becomes truncated by a translational frameshift that adds one unique residue before encountering a stop codon in the −1 reading frame (11–13). This frameshift is highly efficient and occurs approximately 50% of the time.

Organisms other than *E. coli* lack the equivalent of the τ “glue” function, and the three components (DNA polymerase, clamp, and clamp loader) appear to behave as independent units. For example, in eukaryotes from yeast to humans and in bacteriophage T4, the three replicase components do not form a holoenzyme in solution and purify independent of each other (for review, see Ref. 3). Further, frameshifting has not been observed in these dnaX homologs, nor is a frameshift site like that in *E. coli* dnaX contained in the dnaX homolog of the...
Gram-positive *B. subtilis*. Hence, the presence of both T- and γ-like proteins in *T. thermophilus* suggests that its replicase may be organized into a mutiprotein particle similar to the *E. coli* DNA polymerase III holoenzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA modification enzymes and unlabeled nucleotides were from New England Biolabs Inc. Labeled nucleotides were from Amersham Corp. The Alter-1 vector was from Promega. pET plasmids and *E. coli* strains BL21(DE3) and BL21(DE3)pLysS were from Novagen. Oligonucleotides were from Operon Technologies, Inc.

**Genomic DNA—** *T. thermophilus* (strain HB8) was obtained from the American Type Culture Collection. Genomic DNA was prepared from cells grown in 0.1 liter of Thermus medium N697 (4 g of yeast extract, 8.9 g of polypeptide (BDL 11910), 2.0 g of NaCl, 30.0 g of agar, 1 liter of distilled water) at 75 °C overnight. Cells were collected by centrifugation at 4 °C, and the cell pellet was resuspended in 25 ml of 100 mM Tris-HCl (pH 8.0), 0.05 M EDTA, 2 mg/ml lysozyme and incubated at room temperature for 10 min. Then 25 ml of 0.1 M EDTA (pH 8.0), 6% SDS was added and mixed, followed by 60 ml of phenol. The mixture was shaken for 40 min, followed by centrifugation at 10,000 × g for 10 min at room temperature. The upper phase (50 ml) was removed and mixed with 50 ml of phenol/chloroform (50:50 v/v) for 30 min, followed by centrifugation for 10 min at room temperature. The upper phase was decanted, and the DNA was precipitated upon addition of 1/10th volume of 2 M sodium acetate (pH 6.5) and 1 volume of ethanol. The precipitate was collected by centrifugation and washed twice with 2 ml of 80% ethanol, dried, and resuspended in 1 ml of Tris-EDTA buffer.

Oligonucleotide DNA dinucleotides for amplification of the *T. thermophilus* genomic DNA were as follows. The upstream 32-mer, 5′-CCGAAATCTCAGCCTACTCTTCTGCGGAC-3′ (S indicates a mixture of G and C), consists of a HindIII site within the first 9 nucleotides (underlined), followed by codons encoding the subsequent following sequence (HAY-LFSGT). The downstream 34-mer, 5′-CCGGAATCTTGTTGCGTGG- GCCCTCTAGAGGCT-3′, consists of an EcoRI site (underlined), followed by codons encoding the sequence KTLEPPEH on the complementary strand. The amplification reactions contained 10 ng of *T. thermophilus* genomic DNA, 0.5 μm each primer, in a volume of 100 μl of Vent polymerase reaction mixture according to the instructions of the manufacturer (10 μl of ThermoPol buffer, 0.5 μm each dNTP, and 0.5 mM MgSO₄). Amplification was performed using the following cycling scheme: 5 cycles of 30 s at 95.5 °C, 30 s at 40 °C, and 2 min at 72 °C; 5 cycles of 30 s at 95.5 °C, 30 s at 45 °C, and 2 min at 72 °C; 30 cycles of 30 s at 95.5 °C, 30 s at 50 °C, and 2 min at 72 °C; and 30 cycles of 30 s at 95.5 °C, 30 s at 50 °C, and 30 s at 72 °C. Products were visualized in a 1.5% native agarose gel.

Genomic DNA was digested with either *XhoI*, *XbaI*, *Stul*, *PstI*, *Neol*, *MolI*, *KpnI*, *HindIII*, *EcoRI*, *EagI*, or *BamHI*, followed by Southern analysis in a native agarose gel (26). The filter was probed with the PCR2 product radiolabeled by random priming. The sequence from the region downstream of the stop codon (primer sequence: 5′-ggg cga atg cag tcg ggg ggc ggc g-3′) was ligated into *PvuII* fragment of pALTERdnaX, and placing it into *SmaI*/*XbaI*-digested Puc19 to yield Puc19AdnaX. The N-terminal sequence of the *T. thermophilus* dnaX homolog was then reconstructed to position an NdeI site at the N terminus. This was performed by amplifying the 5′ region encoding the N-terminal section of the γ-like proteins using an upstream primer containing an NdeI site that hybridizes to the dnaX homolog at the start codon. The primer sequence for the 5′ end was 5′-ggtgcgtgatgcaaaacgtg-3′, 0 frame shift, total 16-mer (where the NdeI site is underlined and the coding sequence of dnaX follows). The downstream primer hybridizes past the *PmlI* site at nucleotide positions 987–1004 downstream of the start codon (primer sequence: 5′-gggtggtgtgcccagggcactgaa-3′, where the internal 12 nucleotides contain a SfiI/GI restriction site (underlined), followed by the sequence from the region downstream of the stop codon). The 1.9-kb nucleotide extension fragment was digested either with *PmlI* or *NdeI*. *PmlI*/*NdeI* fragment was ligated into *NdeI*/*PmlI*-digested Puc19AdnaX to form Puc19AdnaX. The Puc19AdnaX plasmid was then digested with *NdeI* and *SfiI*, and the 1.9-kb fragment containing the dnaX homolog was purified using the Sephaglas BandPrep Kit (Pharmacia-LKB). pET16b was digested with *NdeI* and *XhoI*. Then the full-length dnaX homolog was ligated into the digested pET16b to form pET16dnaX.

**Purification of T. thermophilus γ- and γ-like Proteins**—Six liters of BL21(DE3)pLysSPETdnaX cells were grown in LB media containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol at 37 °C to an *A₅₆₀* of 0.8, and then IPTG was added to a concentration of 2 mm. After a further 2 h at 37 °C, cells were harvested by centrifugation and stored at −70 °C. The following steps were performed at 4 °C. Cells (150 g, wet weight) were thawed and suspended in 45 ml binding buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol) containing 0.5 M NaCl (Fraction II, 36 g) of 5% Polyvinyl (P) Sigma) was added. Cell debris was removed by centrifugation at 18,000 rpm for 30 min in a Sorvall SS34 rotor at 4 °C. The supernatant (Fraction I, 40 ml, 376 mg of protein) was applied to a 5 ml HiTrap chelating Sepharose column (Pharmacia-LKB). The column was washed with 25 ml of binding buffer, then with 30 ml of binding buffer containing 500 mM imidazole, and then eluted with 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5). Fractions of 1 ml were collected and analyzed on an 8% Coomassie Blue-stained SDS-polyacrylamide gel. Fractions containing subunits migrating at the *T. thermophilus* γ- and γ-like positions, and exhibiting cross reactivity with antibody to *E. coli* γ and γ in a Western analysis, were pooled and dialyzed against buffer A (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM dithiothreitol, and 10% glycerol) containing 0.5 mM NaCl (Fraction II, 36 g).

**References**

1. The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TBS-T, Tris-buffered saline with Tween; TLC, thin layer chromatography.
mg in 7 ml). Fraction II was diluted 2-fold with buffer A and passed through a 2-ml ATP agarose column equilibrated in buffer A containing 0.2 M NaCl to remove any E. coli γ complex contaminant. Then 0.18 mg (300 μl of Fraction II) was gel filtered on a 1-ml Superose 12 column (Pharmacia-LKB) in buffer A containing 0.5 M NaCl. After the first 216 drops, fractions of 200 μl were collected (Fraction III) and analyzed by Western analysis, by ATPase assays, and by Coomassie Blue staining of an 8% SDS-polyacrylamide gel.

**Polyclonal Antibody to E. coli γ/τ—** E. coli γ protein was prepared as described (22). Pure γ protein (100 μg) was brought up in Freund’s adjuvant, and a rabbit polyclonal antibody directed against E. coli γ was then electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell). For molecular size markers, the Kaleidoscope markers (Bio-Rad) were used to visually verify that transfer of proteins onto the membrane had occurred. Membranes were blocked using 5% non-fat milk, washed with 0.05% Tween in Tris-buffered saline (TBS-T) and then incubated for over 1 h with a 1:5000 dilution of rabbit polyclonal antibody directed against E. coli γ and then cut from the TLC sheet and quantitated by liquid scintillation. The extent of ATP hydrolyzed was used to calculate the mol of ATP released/mol of protein/min. One mol of E. coli γ was calculated assuming a mass of 71 kDa/monomer. The ATPase assays were performed in 0.5 ml of 20 mM Tris-Cl (pH 7.5), 8 mM MgCl₂ containing 0.72 μg of M13mp18 single-stranded DNA (where indicated), 100 μM γ[32P]ATP (specific activity of 2000–4000 cpm/pmol), and the indicated protein. Some reactions contained additional NaCl where indicated. Reactions were incubated at the temperatures indicated in the legend to Fig. 11 for 30 min and then were quenched with an equal volume of 25 mM EDTA. The aliquots were analyzed by spotting them (1 μl each) onto thin layer chromatography (TLC) sheets coated with Cel-300 polyethyleneimine (Brinkmann Instruments Co.). TLC sheets were developed in 0.5% gelatin in TBS-T at room temperature. Membranes were washed using TBS-T buffer, and then antibody was detected on x-ray film (Kodak) using the ECL kit from Amersham Corp. The samples in- Using TBS-T buffer, and then antibody was detected on x-ray film (Kodak) using the ECL kit from Amersham Corp. The samples in-

**RESULTS**

**Identification of the T. thermophilis dnaX Homolog**—The dnaX genes of the Gram-negative E. coli and the Gram-positive B. subtilis share more than 56% identity in amino acid sequence within the N-terminal 180 residues containing the ATP-binding domain (Fig. 1). Two highly conserved regions (shown in bold in Fig. 1) were used to design oligonucleotide primers for application of the polymerase chain reaction to T. thermophilis genomic DNA. Use of these primers with genomic T. thermophilis DNA resulted in a product of the expected size (345 nucleotide base pairs). The PCR product was radiolabeled and used to probe genomic DNA in a Southern analysis (Fig. 2).

The Southern analysis showed a single XbaI fragment of approximately 4 kb, more than sufficient length to encode the dnaX homolog. An XbaI digest of genomic DNA was ligated into XbaI-digested Alter-1 vector. Ligated DNA was transformed into DH5α cells, and colonies were screened with the labeled PCR probe. The sequence of the dnaX homolog contained within the insert is shown in Fig. 3.

**Analysis of the T. thermophilis dnaX Homolog**—The XbaI insert encodes a large open reading frame shown in Fig. 2. There are two possible in-frame candidate GTG codons. The first is 30 residues upstream of the second start site shown in Fig. 3 (this site is present in the Fig. 3 sequence upstream of the start site of the translated protein sequence of Fig. 3). The N-terminal region of γ/τ is homologous in several bacteria, both Gram-positive and -negative (Fig. 4). Use of the first start site would lead to a product having extra residues attached to its N terminus compared with the other dnaX homologs. The second start site, shown in Fig. 3, would produce a protein with a similar N-terminal sequence to the dnaX products of other bacteria. Indeed, as explained later, expression of the protein from the second GTG codon yields a protein having biochemical properties expected of products of dnaX. Nonetheless, it is still possible that the upstream codon is used. We are attempting to purify the DNA polymerase III holoenzyme from T. thermophilis for N-terminal analysis of the τ protein to resolve this ambiguity.

Assuming that the T. thermophilis dnaX homolog starts with the second GTG codon, as shown in Fig. 3, the resulting protein consists of 529 amino acids (58.0 kDa), as compared with the full-length B. subtilis (62.7 kDa mass) (28) and E. coli (71.1 kDa) (29) dnaX gene products. The consensus GXXGXXKT motif for nucleotide binding is conserved in all of these protein products. The dnaX homolog-encoded protein products also contain four Cys residues that form a putative zinc finger (marked with asterisks in Fig. 4). This putative zinc finger does not match the consensus site for zinc binding, but the crystal structure of δ reveals one atom of zinc coordinated to four Cys residues (10). δ is a protein subunit of the γ complex clamp loader and is homologous to the γ and τ proteins (15). These Cys residues that participate in coordinating zinc in δ are conserved in the E. coli γ/τ proteins. Indeed, the E. coli γ and τ proteins bind one atom of zinc.³

Overall, the level of amino acid identity of the T. thermophilis dnaX homolog to both E. coli dnaX and the B. subtilis dnaX homolog in the N-terminal 165 residues is 53%. After this

region of identity, the C-terminal region of the *T. thermophilis* dnaX homolog shares 26% and 20% identity to *E. coli* dnaX gene and the *B. subtilis* dnaX homolog, respectively. A proline-rich region, downstream of the conserved region, is also present in the *T. thermophilis* dnaX homolog (residues 346–375) but not in the *B. subtilis* dnaX homolog (see Fig. 3). The overall identity between *E. coli* dnaX and the *T. thermophilis* dnaX homolog over the entire gene is 34%. Identity of the *T. ther-

---[**Fig. 3.** Sequence of the *T. thermophilis* dnaX homolog. The DNA sequence (uppercase) and predicted amino acid sequence (lowercase) of the *T. thermophilis* dnaX homolog yields a 529-amino acid protein (r-like protein) of 58.0 kDa. A putative frameshifting sequence containing several A residues 1478–1486 (underlined) results in a smaller protein (g-like protein) of 49.8 kDa. The potential Shine-Dalgarno (S.D.) signal is bold and underlined. The start codon is bold, and the stop codon for the r-like protein is marked by an asterisk. The potential stop codon for the g-like protein is shown in bold after the frameshift site, and two potential Shine-Dalgarno sequences upstream of the frameshift site are indicated. The ATP binding site is indicated, and the asterisks above the four Cys residues near the ATP site are shown. The potential Zn2 finger is indicated above the sequence. Numbering of the nucleotide sequence is presented at the right. Numbering of the amino acid sequence of the g-like protein is shown in parentheses at the right.}
mophilis and B. subtilis dnaX homolog over the entire gene is 28%.

The Frameshift Site—The dnaX gene of E. coli supports an efficient -1 translational frameshift resulting in approximately equal amounts of two proteins, γ and τ (11–13). The τ protein is the full-length product. The γ protein is the product of a -1 frameshift, which occurs after 2/3 of the gene has been translated and results in addition of one unique amino acid before encountering a stop codon to produce γ. The -1 frameshift site in the E. coli dnaX gene contains the sequence, A AAA around the frameshifting site, including the slippery sequence (11–13). Immediately downstream of the stop codon is a potential stem-loop structure that enhances frameshifting, presumably by causing the ribosome to pause. Further, the AAG codon lacks a cognate tRNA before encountering a stop codon to produce γ. The frameshifting step loop structures immediately downstream. Finally, there is a Shine-Dalgarno sequence immediately adjacent to the frameshift site as well as another Shine-Dalgarno sequence 22 nucleotides upstream of the frameshift site. Assuming the first stop codon is utilized (i.e. -2 frameshift), the predicted size of the T. thermophilis dnaX homolog is 454 amino acids (49.8 kDa), 23 residues longer than E. coli. Use of a +1 frameshift in E. coli results in the addition of 23 amino acids (49.8 kDa), 23 residues longer than E. coli. Use of a +1 frameshift prior to the stop codon. Use of a +1 frameshift would result in 12 additional residues for a molecular mass of 50.8 kDa. As explained later, this nucleotide sequence was found to promote both -1 and -2 frameshifting in E. coli (Fig. 7). But first, we examined T. thermophilis cells by Western analysis for the presence of two proteins homologous to E. coli γ and τ.

Western Analysis of T. thermophilis Cells for Presence of γ and τ-Proteins—The high level of identity between the B. subtilis N-terminal regions of the products of the M. genitalium, M. gen. homolog gene sequence, A AAA and B. subtilis, Bacillus subtilis; C. cres., Caulobacter crescentus; M. gen., Mycoplasma genitalium; T. th., Thermus thermophilis. Alignments were produced using Clustal.
thermophilis cells lysed in SDS. The results show that the antibody is rather specific for two high molecular proteins that migrate in the vicinity of the molecular masses of E. coli \(\gamma\) and \(\tau\).

The T. thermophilis dnaX Homolog Supports a Translational Frameshift—The presence of two proteins in T. thermophilis cells that cross-react with antibody directed against E. coli \(\gamma\) indicates that the T. thermophilis dnaX homolog supports a translational frameshift that produces both \(\gamma\)- and \(\tau\)-like proteins. To gain further support for frameshifting in the T. thermophilis dnaX homolog, the putative frameshifting sequence was analyzed for ability to promote frameshifting in E. coli. A region surrounding the T. thermophilis dnaX homolog frameshift site was inserted into the \(\beta\)-galactosidase gene of pUC19 in three different reading frames (stop codons were mutated where needed to prevent stoppage following a frameshift). These three plasmids were introduced into E. coli and plated on X-gal. The results, in Fig. 7, show blue colonies with all three plasmids, and therefore, the T. thermophilis frameshifting sequence supports both \(-1\) and \(-2\) frameshifting.

To further these results, two G residues were substituted for A residues in the frameshift site to disrupt the ability of this sequence to direct frameshifting. The mutated frameshifting sequence was inserted into pUC19 in all three reading frames, transformed into E. coli cells, and plated on LB plates containing X-gal. The frameshift sequence was also mutated by inserting two Gly residues into the site and then cloned into pUC19 in all three reading frames. Blue colonies are indicated by the plus signs. The picture shows the colonies, and the type of frameshift required for read through (blue color) is indicated next to the sector.

Expression and Purification of \(\gamma\)- and \(\tau\)-like Proteins—The dnaX homolog was engineered into the T7-based IPTG-inducible pET16 vector as illustrated in Fig. 8 (the second GTG codon was used as shown in Fig. 3). This should produce a protein containing the sequence of \(\gamma\)- and \(\tau\)-like proteins, along with a 21-residue leader containing 10 contiguous His residues (tagged-\(\tau\) = 60.6 kDa; tagged-\(\gamma\) = 52.4 kDa, assuming a \(-2\) frameshift). The pETdnaX plasmid was introduced into BL21(DE3)pLysS cells harboring the gene encoding T7 RNA polymerase under control of the lac repressor. Log phase cells were induced with IPTG and analyzed before and after induction in an SDS-polyacrylamide gel (Fig. 9A). The result shows that upon induction, two new proteins are expressed with the approximate sizes expected of the T. thermophilis \(\gamma\) and \(\tau\) subunits (larger than E. coli \(\gamma\) and smaller than E. coli \(\tau\)). The two proteins are produced in nearly equal amounts, similar to the case of the E. coli \(\gamma\) and \(\tau\) subunits. Western analysis using antibodies against the E. coli \(\gamma\) and \(\tau\) subunits cross-reacted with the induced proteins, further supporting their identity as T. thermophilis \(\gamma\)- and \(\tau\)-like proteins (data not shown, but repeated with the pure subunits shown in Fig. 9C).

The His-tagged T. thermophilis \(\gamma\)- and \(\tau\)-like proteins were purified from 6 liters of induced E. coli cells containing the pETdnaX plasmid. Cells were lysed and clarified from cell debris by centrifugation, and the supernatant was applied to a HiTrap chelate affinity column. Elution of the chelate affinity mixture of T. thermophilis \(-\) and \(+\) tetramers (20). The \(\gamma\)- and \(\tau\)-like proteins are present in nearly equal amounts.

The \(\gamma\)- and \(\tau\)-like proteins were further purified by gel filtration on a Superose 12 column (Fig. 9B, and Fig. 10). Recovery of T. thermophilis \(-\) and \(+\)-like proteins through gel filtration was 81%. A mixture of E. coli \(\gamma\)-proteins results in a mixed tetramer of \(\gamma_2\tau_2\) along with \(\tau_4\) and \(\gamma_4\) tetramers (20). The mixture of T. thermophilis \(\gamma\)-\(\tau\)-like proteins elutes ahead of the
A genomic fragment containing a partial sequence of the dnaX homolog was cloned into pALTER-1. This fragment was subcloned into pUC19 (pUC19ΔdnaX). Then the N-terminal section encoded by the dnaX homolog was amplified such that the fragment was flanked by NdeI (at the initiating codon) and the internal BamHI site. This fragment was inserted to form the entire coding sequence of the dnaX homolog in pUC19 (pUC19ΔdnaX). The dnaX homolog was then cloned behind the polyhistidine leader in the T7 based expression vector pET16 to give pET16 dnaX. Details are under “Experimental Procedures.”

Characterization of the γ- and τ-like Proteins—The E. coli τ protein is a DNA-stimulated ATPase (33, 34). The γ protein binds ATP but does not hydrolyze it even in the presence of DNA unless other proteins of the DNA polymerase III holoenzyme are also present (35). Next we examined the T. thermophilis γ/τ-like proteins for DNA-stimulated ATPase activity. The γ/τ-like proteins exhibited DNA-stimulated ATPase activity (Fig. 11, top panel). The specific activity of the T. thermophilis γ/τ-like proteins was 11.5 mol of ATP hydrolyzed/mol γ/τ (as monomer and assuming an equal mixture of the two). Furthermore, analysis of the gel filtration column fractions shows that the ATPase activity co-elutes with the T. thermophilis γ/τ-like proteins, supporting evidence that this weak ATPase activity is intrinsic to the γ/τ-like proteins (Fig. 10). The specific activity of the γ/τ-like proteins before gel filtration was the same as after gel filtration (within 10%), further indicating that the DNA-stimulated ATPase is an inherent activity of the γ/τ-like proteins. Presumably, only the τ-like protein contains ATPase activity (as τ)

FIG. 8. Construction of the T. thermophilis dnaX homolog expression vector. A genomic fragment containing a partial sequence of the dnaX homolog was cloned into pALTER-1. This fragment was subcloned into pUC19 (pUC19ΔdnaX). Then the N-terminal section encoded by the dnaX homolog was amplified such that the fragment was flanked by NdeI (at the initiating codon) and the internal BamHI site. This fragment was inserted to form the entire coding sequence of the dnaX homolog in pUC19 (pUC19ΔdnaX). The dnaX homolog was then cloned behind the polyhistidine leader in the T7 based expression vector pET16 to give pET16 dnaX. Details are under “Experimental Procedures.”
activity, as in the case of E. coli. Assuming only the T. thermophilis \( \tau \)-like protein contains ATPase activity, its specific activity is twice the observed rate (after factoring out the weight of the \( \gamma \)-like protein).

The ATPase activity is lower at \( 37^\circ \)C than at \( 65^\circ \)C (Fig. 11, middle panel), consistent with the activity behavior of proteins isolated from a thermophile source. However, there is no apparent increase in activity in proceeding from 50 to \( 65^\circ \)C (the rapid breakdown of ATP above \( 65^\circ \)C precluded measurement of ATPase activity at temperatures above \( 65^\circ \)C). In contrast, the E. coli \( \tau \) subunit lost most of its ATPase activity upon elevating the temperature to \( 50^\circ \)C (Fig. 11, middle panel). These reactions contain no stabilizers such as a nonionic detergent or gelatin, nor did they include substrates such as ATP, DNA, or magnesium.

Last, we examined the relative stability of T. thermophilis \( \gamma/\tau \)-like proteins and the E. coli \( \gamma/\tau \) proteins to the addition of NaCl (Fig. 11, bottom panel). Whereas the E. coli \( \tau \) subunit rapidly lost activity at even 0.2 M NaCl, the T. thermophilis \( \gamma/\tau \) retained full activity in 1.0 M NaCl and was still 80% active in 1.5 M NaCl.

**DISCUSSION**

This report identifies a homolog of the E. coli dnaX gene of *T. thermophilis*. Like the E. coli gene, the *T. thermophilis* dnaX homolog encodes two related proteins through use of a highly efficient translational frameshift. Furthermore, the \( \gamma/\tau \)-like proteins display DNA-stimulated ATPase activity. As expected for proteins from a thermophile, the ATPase activity of the \( \gamma/\tau \)-like proteins is thermostable and resistant to elevated concentrations of salt.

The translational frameshifting sequence of the *T. thermophilis* dnaX homolog is significantly different from that in *E. coli*. In *E. coli*, the heptamer frameshifting site contains six A residues followed by a G residue in the context A AAA AAG. This sequence satisfies the XXY YYY rule for \(-1 \) frameshifting (30). The frameshift is made more efficient by the absence of the AAG tRNA for Lys, which presumably leads to stalling of the ribosome at the frameshifting site and increases the efficiency of frameshifting (31). Two additional aids to frameshifting include a downstream hairpin and an upstream Shine-Dalgarno sequence (11, 32).

In *T. thermophilis*, the dnaX homolog frameshifting heptamer is A AAA AAA; it is flanked by two other Ala residues, one on each side, and a downstream region of secondary structure. The nearest downstream stop codon is positioned such that only two residues would be added following the frameshift, as in *E. coli*. However, this stop codon is in the \(-2 \) reading frame. There is no precedent in nature for \(-2 \) frameshifting, although \(-2 \) frameshifting has been shown to occur in test cases (27). Analysis of the *T. thermophilis* frameshifting sequence in *E. coli* shows that this natural sequence promotes both \(-1 \) and \(-2 \) frameshifting. A \(-1 \) frameshift in the *T. thermophilis* dnaX homolog would result in a C-terminal extension of 12 residues prior to the stop codon. At present, the results do not discriminate between which path occurs in *T. thermophilis*, a \(-1 \) or \(-2 \) frameshift, or a combination of the two.

An upstream Shine-Dalgarno sequence stimulates frameshifting in *E. coli* (for review, see Ref. 27, 36). In release factor 2 (RF2), the Shine-Dalgarno sequence is three nucleotides upstream of the \(+1 \) frameshift site. In the case of *E. coli* dnaX, the Shine-Dalgarno sequence is positioned ten nucleotides upstream of the \(-1 \) frameshift site. In the *T. thermophilis* dnaX homolog, there are two Shine-Dalgarno sequences just upstream of the frameshift site. One is immediately adjacent to the site, and the other is 22 residues upstream (see Fig. 3). To determine which of these Shine-Dalgarno sequences plays a role in frameshifting, if any, will require further study.

The association between the *E. coli* \( \gamma/\tau \) subunits forms the central contact point that holds together the large DNA polymerase III holoenzyme containing two DNA polymerases and one clamp loader (20). This organized superstructure confers onto the holoenzyme the ability to simultaneously replicate both strands of duplex DNA in a highly coordinated fashion (23–25). However, it appears that most organisms do not contain a holoenzyme forming the replicase. For example, the clamp loader and polymerase of the well studied human and yeast replication systems are not organized into a holoenzyme structure. Further, the polymerase and clamp loader of the bacteriophage T4 replicase do not associate in solution. Presumably, these replicases form a holoenzyme upon their association onto DNA.

The presence of a \( \gamma \)-like protein in *T. thermophilis* suggests it has a clamp loading apparatus and thus a clamp as well. Although further study is needed to determine the subunit structure of the *T. thermophilis* replicase, the presence of the \( \gamma \)-like protein implies a replicative polymerase with a structure similar to that of *E. coli* DNA polymerase III holoenzyme.
Acknowledgments—We thank Peter Model and Marjorie Russel for encouragement in this project.

REFERENCES
NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

*Thermus thermophilus dnaX* Homolog Encoding γ- and τ-like Proteins of the Chromosomal Replicase

Olga Yurieva, Maija Skangalis, John Kuriyan and Mike O'Donnell

doi: 10.1074/jbc.272.43.27131

Access the most updated version of this article at [http://www.jbc.org/content/272/43/27131](http://www.jbc.org/content/272/43/27131)

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 23 of which can be accessed free at [http://www.jbc.org/content/272/43/27131.full.html#ref-list-1](http://www.jbc.org/content/272/43/27131.full.html#ref-list-1)