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 act 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 (αεθ), the β subunit DNA sliding clamp, and the five protein γ complex clamp loader (γδφ χθφ) (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 χψ 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 τγ 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 (τγδψφ) 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

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To whom correspondence should be addressed: 1230 York Ave., New York, NY 10021. Tel.: 212-327-7251; Fax: 212-327-7254.

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1 J. Gulbis and J. Kuriyan, unpublished observations.
Gram-positive *B. subtilis*. Hence, the presence of both \( \tau \) and \( \gamma \)-like proteins in *T. thermophilis* suggests that its replicase may be organized into a multimeric protein 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. \( \Phi \) PstI plasmids and *E. coli* strains BL21(DE3) and BL21(DE3)pLy85 were from Novag. Oligonucleotides were from Operon Technologies, Inc.

**Genomic DNA—** *T. thermophilis* (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 polypeptone (Difco 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 mM EDTA, 2 mg/ml lysozyme and incubated at room temperature for 10 min. Then 25 ml of 0.1 mM 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 \( \times \) 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 10% 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.

**Oligonucleotides**—The DNA oligonucleotides for amplification of *T. thermophilis* genomic DNA were as follows. The upstream 32-mer, 5' -GCCAGAATTCGCTACCTTCTTCTCCGASAC-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 following sequence (HAY-LFSGT). The downstream 34-mer, 5'-CGAATTCGCTACCTTCTTCTCCGASAC-3', consists of an EcoRI site (underlined), followed by codons encoding the sequence KTLEPEPEH on the complementary strand. The amplification reactions contained 10 ng of *T. thermophilis* genomic DNA, 0.5 \( \mu \)M each primer, in a volume of 100 \( \mu \)l of Vent polymerase reaction mixture according to the instructions of the manufacturer (10 \( \mu \)l of Thermopol buffer, 0.5 \( \mu \)M each dNTP, and 0.5 \( \mu \)M MgSO\textsubscript{4}). 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; 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, *Stu*I, *Pst*I, NcoI, *Mlu*I, *Kpn*I, HindIII, EcoRI, *Bgl*II, or BamHI, followed by Southern analysis in a native agarose gel (26). The probe was filtered with the PCR\textsuperscript{2} product radiolabeled with random priming. *XhoI* digested genomic DNA showed a single 4-kb fragment. A genomic plasmid library containing XhoI inserts was prepared upon digesting 1 \( \mu \)g of genomic *T. thermophilis* DNA with 10 units of XhoI in 100 \( \mu \)l of NEBuffer N2 (50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM MgCl\textsubscript{2}, 1 mM dithiothreitol) for 2 h at 37 °C. The Alter-1 vector (0.5 \( \mu \)g) (Promega) was digested with 1 unit of XhoI in NEBuffer N2. After purification of the XhoI digests by phenol/chloroform extraction and ethanol precipitation, the genomic digest was incubated with 0.05 \( \mu \)g of digested Alter-1 and 20 units of T4 ligase in 30 \( \mu \)l of ligase buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, and 1 mM ATP) at 15 °C for 12 h. The ligation reaction was transferred into the DH\textsubscript{5a} strain of *E. coli*, and transformants were plated on LB plates containing ampicillin and screened for the *dnaX* insert using the radiolabeled PCR probe. Plasmid DNA was prepared from 20 positive colonies, and of these, 6 contained the expected 4-kb insert when digested with XhoI. Sequencing of the insert was performed by the Sanger method using the Vent polymerase and a 20 \( \mu \)l aliquot of the radiolabeled primer. The upstream primer for the mutant frameshift site was: 5'-ggg cga att cgc gct tcg gga ggt ggg-3'. The downstream 34-mer, 5'-ggg cga att cgc gct tcg gga ggt ggg-3', was designed to frame-shift the 5'-extension to the 0 frame insert was blunt ended to produce the greater length insert (converting the *EcoRI* site to an attntaatt sequence). The taq sequence, which produces the -1 frame tga stop codon was mutated to tgg in the downstream primer (the frameshift site was then placed after the frameshift occurred). The six plasmids were transformed into *E. coli* strain DH5\textalpha{} and plated onto LB plates containing ampicillin and X-gal. The appearance of blue color was scored after 24 h of incubation at 37 °C.

**Expression Vector for T. thermophilis \( \gamma \) and \( \gamma \)-like Proteins**—The *T. thermophilis* dnaX homolog was cloned into the \( \Phi \)ET16 expression vector in two steps. First, the bulk of the gene was cloned into \( \Phi \)ET16 by removing the PmI/XbaI fragment from pPmI/tnaX and placing it into *SmaI/XbaI-digested Puc19* to yield Puc19 dnaX. The N-terminal sequence of the *T. thermophilis* dnaX homolog was then reconstructed to position an Ndel site at the N terminus. This was performed by amplifying the 5' region encoding the N-terminal portion of the \( \gamma \)-like proteins using an upstream primer containing an Ndel site that hybridizes to the *T. thermophilis* dnaX homolog at the start codon. The primer sequence for the 5' end was 5'-gtggtgctatg gtg agc gcc ctc tac cgc c-3'. The downstream primers have an RI site (underlined and the coding sequence of *T. thermophilis* dnaX in a Western analysis, were pooled and washing with 25 ml of binding buffer, then with 30 ml of binding buffer HiTrap chelating Sepharose column (Pharmacia-LKB). The column was supernatant (Fraction I, 40 ml, 376 mg of protein) was applied to a 5-ml Polymin P (Sigma) was added. Cell debris was removed by centrifugation.

**Replication Accessory Proteins of Thermus thermophilis**

The upstream primer contained the heptamer site (5'-gcg cgg atc cgg agg gag aaa aaa gca tca ca-3'). The BamHI site for cloning into pUC19 is as follows: 5'-gaa tta tat tgc ggc tgg ggt ggt ggt ggt g-3'. The downstream primers had the frameshift site then placing this insert into the three reading frames of the *T. thermophilis* gene as described above. Production of the PCR inserts containing the frameshift site were conducted as follows. The upstream primer contained 5'-gcg cga att cgc gct tcg gga ggt ggg-3', -1 frame, 54-mer insert; and 5'-gcg cga att cgc gct tcg gga ggt ggg-3', -2 frame, 56-mer insert. The downstream primers have an *EcoRI* site (underlined); the *EcoRI* site of the 0 frame insert was blunt ended to produce the greater length insert (converting the *EcoRI* site to an attntaatt sequence). The taq sequence, which produces the -1 frame tga stop codon was mutated to tgg in the downstream primer (the frameshift site was then placed after the frameshift occurred). The six plasmids were transformed into *E. coli* strain DH5\textalpha{} and plated onto LB plates containing ampicillin and X-gal. The appearance of blue color was scored after 24 h of incubation at 37 °C.
Asterisks indicate identities. The ATP binding consensus sequence is indicated. The two regions used for PCR primer design are shown in bold.

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 3-bar 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.

Polyconal 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 injected subcutaneously into a New Zealand Rabico (Rabbit Farms). After 2 weeks, a booster consisting of 50 μg γ in Freund’s adjuvant was administered, followed after 2 weeks by a third injection (50 μg).

Western Analysis of T. thermophilis γ/τ-like Proteins—Western analysis of samples were performed in duplicate 10% SDS-polyacrylamide gels. One gel was Coomassie stained to evaluate the pattern of proteins and the other gel was then electroblotted 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 in 1% 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 included: 1) a mixture of E. coli γ (15 ng) and τ (15 ng) subunits; 2) T. thermophilis whole cells (100 μl) suspended in cracking buffer; and 3) purified T. thermophilis γ and τ fraction II (0.6 μg as a mixture).

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 50% 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 τ'γ 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 GXGGXGKT 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

3 J. Turner and M. O'Donnell, unpublished atomic adsorption results.
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 (∼108 kDa). A putative frameshifting sequence containing several A residues (1478–1486) results in a smaller protein (131-amino acid 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 r-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 indicate the putative Zn$^{2+}$ finger. The proline-rich area is indicated above the sequence. Numbering of the nucleotide sequence is presented at the right. Numbering of the amino acid sequence of the r-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, AAA...AA (Fig. 6). This "slippery sequence" preserves the initial two residues of the tRNAs in the aminoacyl and peptidyl sites both before and after the frameshift. Mutagenesis of the E. coli dnaX frameshifting site has shown that the first three residues can be nucleotides other than A but that A in the second set of three nucleotides is important to frameshifting (31). 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. -1 frameshift would result in 12 additional amino acids (49.8 kDa), 23 residues longer than the predicted size of the protein (47.5 kDa). Use of a -1 frameshift results in the addition of two residues (Lys-Ala) after the frameshift prior to the stop codon, thereby facilitating the vigorous frameshifting observed in E. coli dnaX (31). A fourth component of frameshifting in E. coli dnaX is presence of an upstream Shine-Dalgarno sequence, which pairs with 16 S rRNA to increase the frequency of frameshifting still further (32).

Examination of the T. thermophilis dnaX homolog sequence reveals a site that fulfills the X XXX YYY rule in which positions 4–7 are A residues (Fig. 5). The site differs from that in E. coli in that all seven residues are A, and the heptanucleotide is flanked on each side by an additional A residue. Surprisingly, T. thermophilis dnaX contains an additional A both upstream and downstream of the sites. Indeed, a -2 frameshift would still accommodate the requirement that the first two nucleotides of each codon in the peptidyl and aminoacyl sites be conserved. As with the case of E. coli dnaX, there are secondary structure 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 γ-like protein is 454 amino acids (49.8 kDa), 23 residues longer than E. coli γ protein (47.5 kDa). Use of a -2 frameshift results in the addition of two residues (Lys-Ala) after the frameshift, thereby facilitating the vigorous frameshifting observed in E. coli dnaX (31). A fourth component of frameshifting in E. coli dnaX is presence of an upstream Shine-Dalgarno sequence, which pairs with 16 S rRNA to increase the frequency of frameshifting still further (32).
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 γ and 7.

The T. thermophilis dnaX Homolog Slippery Sequence Supports Both −1 and −2 Frameshifting—The presence of two proteins in T. thermophilis cells that cross-react with antibody directed against E. coli γ indicates that the T. thermophilis dnaX homolog supports a translational frameshift that produces both γ- and τ-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 β-galactosidase gene of pUC19 in the 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 in the presence of 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 followed by transformation into E. coli and plating on X-gal. Now the results show that both −1 and −2 frameshifting is prevented, further supporting the identity of the frameshift site (Fig. 7).

Expression and Purification of γ- and τ-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 γ- and τ-like proteins, along with a 21-residue leader containing 10 contiguous His residues (Tagged-τ = 60.6 kDa; Tagged-γ = 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 γ and τ subunits (larger than E. coli γ and smaller than E. coli τ). The two proteins are produced in nearly equal amounts, similar to the case of the E. coli γ and τ subunits. Western analysis using antibodies against the E. coli γ and τ subunits cross-reacted with the induced proteins, further supporting their identity as T. thermophilis γ- and τ-like proteins (data not shown, but repeated with the pure subunits shown in Fig. 9C).

The His-tagged T. thermophilis γ- and τ-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 column yielded approximately 35 mg of protein containing two bands that migrated in a region, consistent with the molecular weight predicted from the dnaX homolog (Fig. 9B), and produced a positive signal by Western analysis using polyclonal antibody directed against the E. coli γ and τ proteins (Fig. 9B). The γ- and τ-like proteins are present in nearly equal amounts.

The γ- and τ-like proteins were further purified by gel filtration on a Superose 12 column (Fig. 9B, and Fig. 10). Recovery of T. thermophilis γ- and τ-like proteins through gel filtration was 81%. A mixture of E. coli γ/τ proteins results in a mixed tetramer of γ₂τ₂ along with τ₄ and γ₄ tetramers (20). The mixture of T. thermophilis γ/τ-like proteins elutes ahead of the
150-kDa marker and, thus, is consistent with the mass of τ4 and γ4 tetramers. It is also possible that the mixture of γ- and τ-like proteins produces a γ2τ2 tetramer that would comigrate with τ4 and γ4, although further studies would be needed to address the possibility that they directly interact to form a tetramer.

As described earlier, the dnaX homolog frameshifting sequence could produce either a −1 or −2 frameshift to yield a His-tagged γ subunit of either 53.3 or 52.4 kDa, respectively. The difference in these two possible products is too close to determine from migration in SDS-polyacrylamide gels. It also remains possible that both frameshifting γ-like products are present and do not resolve under the conditions used.

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.
activity, as in the case of *E. coli*. Assuming only the *T. thermophilis* \(\gamma\)/\(\tau\)-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 °C than at 65 °C (Fig. 11, middle panel), consistent with the activity behavior of proteins isolated from a thermophilic source. However, there is no apparent increase in activity in proceeding from 50 to 65 °C (the rapid breakdown of ATP above 65 °C precluded measurement of ATPase activity at temperatures above 65 °C). In contrast, the *E. coli* \(\tau\) subunit lost most of its ATPase activity upon elevating the temperature to 50 °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.

**FIG. 11.** Characterization of the *T. thermophilis* \(\gamma\)/\(\tau\)-like protein ATPase activity. The *T. thermophilis* \(\gamma\)/\(\tau\)-like protein and *E. coli* \(\gamma\)/\(\tau\)-like protein are compared for their ATPase activity characteristics. Due to the greater activity of *E. coli* \(\tau\), the values are plotted as percent for ease of comparison. Actual specific activities for 100% values are given below as pmol of ATP hydrolyzed/30 min/pmol of *T. thermophilis* \(\gamma\)/\(\tau\)-like protein (or pmol of *E. coli* \(\gamma\)/\(\tau\)-like protein). A, *T. thermophilis* \(\gamma\) and \(\tau\)-like protein ATPase is stimulated by the presence of single-stranded DNA. *T. thermophilis* \(\gamma\)/\(\tau\)-like protein was incubated at 65 °C. Specific activity values were 11.5 (+DNA) and 2.5 (–DNA); *E. coli* \(\gamma\)/\(\tau\)-like protein was assayed at 37 °C. Specific activity values were 112.5 (+DNA) and 73.3 (–DNA). B, temperature stability of DNA-stimulated ATPase activity. *T. thermophilis* \(\gamma\)/\(\tau\)-like protein, 11.3 (65 °C); *E. coli* \(\gamma\)/\(\tau\)-like protein, 97.5 (37 °C). C, stability of *T. thermophilis* \(\gamma\)/\(\tau\)-like protein ATPase to NaCl. *T. thermophilis* \(\gamma\)/\(\tau\)-like protein, 8.1 (100 mM added NaCl, and 65 °C); *E. coli* \(\gamma\)/\(\tau\)-like protein, 52.7 (0 mM added NaCl and 37 °C).

**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 XXXY YZX 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 frameshift 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* \(\gamma\) and \(\tau\) 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 form of 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.
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REFERENCES
1. Kornberg, A., and Baker, T. (1992) DNA Replication, 2nd Ed., pp. 165–194, W. H. Freeman and Co., New York
2. Kuriyan, J., and O'Donnell, M. (1993) J. Mol. Biol. 234, 915–925
3. Kelman, Z., and O'Donnell, M. (1994) Curr. Opin. Genet. Dev. 4, 185–195
4. Stukenberg, P. T., Studwell-Vaughan, P. S., and O'Donnell, M. (1991) J. Biol. Chem. 266, 11328–11334
5. O'Donnell, M., Kuriyan, J., Kong, X-P., Stukenberg, P. T., and Onrust, R. (1992) Mol. Biol. Cell 3, 953–957
6. Kelman, Z., and O'Donnell, M. (1995) Annu. Rev. Biochem. 64, 171–200
7. Kong, X-P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992) Cell 69, 425–437
8. Krishna, T. S., Kong, X.-P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) J. Biol. Chem. 269, 11328–11334
9. Guibus, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996) Cell 87, 297–306
10. Guenther, B. D. (1996) Structural Studies on the DNA Replication Apparatus: X-ray Crystal Structure of the S' Subunit of Escherichia coli DNA Pol III. Ph.D. thesis, Rockefeller University
11. Tsuchihashi, Z., and Kornberg, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2516–2520
12. Flower, A. M., and McHenry, C. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3713–3717
13. Blinkowa, A. L., and Walker, J. R. (1990) Nucleic Acids Res. 18, 1725–1729
14. Carter, J. R., Franden, M. A., Aebersold, R., and McHenry, C. S. (1993) J. Bacteriol. 175, 3812–3822
15. Dong, Z., Onrust, R., Skangalis, M., and O'Donnell, M. (1993) J. Biol. Chem. 268, 11768–11765
16. Chen, M., Pan, Z-Q., and Hurwitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5211–5215
17. O'Donnell, M., Onrust, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) Nucleic Acids Res. 21, 1–3
18. Onrust, R., and O'Donnell, M. (1993) J. Biol. Chem. 268, 11766–11772
19. Cullman, G., Fien, K., Kobayashi, R., and Stillman, B. (1995) Mol. Cell. Biol. 15, 4661–4671
20. Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O'Donnell, M. (1995) J. Biol. Chem. 270, 13666–13677
21. McHenry, C. S. (1992) J. Biol. Chem. 267, 2657–2663
22. Studwell-Vaughan, P. S., and O'Donnell, M. (1991) J. Biol. Chem. 266, 19833–19841
23. Maki, H., Maki, S., and Kornberg, A. (1988) J. Biol. Chem. 263, 6570–6578
24. Wu, C. A., Zechnler, E. L., Hughes, A. J., Franden, M. A., McHenry, C. S., and Marians, K. J. (1992) J. Biol. Chem. 267, 4064–4073
25. Yuzhakov, A., Turner, J., and O'Donnell, M. (1996) Cell 86, 877–886
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 76–85 and 382–367, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Weiss, R. B., Dunn, D. M., Atkins, J. F., and Gesteland, R. F., (1987) Cold Spring Harbor Symp. Quant. Biol. 52, 657–698
28. Alonso, J. C., Shirahige, K., and Ogasawara, N. (1990) Nucleic Acids Res. 18, 6771–6777
29. Yin, K-C., Blinkowa, A., and Walker, J. R. (1986) Nucleic Acids Res. 14, 6541–6549
30. Jacks, T., Madhami, H. D., Masiarz, F. R., and Varmus, H. E. (1988) Cell 55, 447–458
31. Tsuichishahi, Z., and Brown, P. O. (1992) Genes Dev. 6, 511–519
32. Larsen, B., Wills, N. M., Gesteland, R. F., and Atkins, J. F. (1994) J. Bacteriol. 176, 6842–6851
33. Lee, S. H., and Walker, J. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2713–2717
34. Tsuichishahi, Z., and Kornberg, A. (1989) J. Biol. Chem. 264, 17790–17795
35. Onrust, R., Stukenberg, P. T., and O'Donnell, M. (1991) J. Biol. Chem. 266, 21681–21686
36. Gesteland, R. F., and Atkins, J. F. (1996) Annu. Rev. Biochem. 65, 741–768
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Olga Yurieva, Maija Skangalis, John Kuriyan and Mike O'Donnell

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