Shuttle Vector Expression in *Thermococcus kodakaraensis*: Contributions of *cis* Elements to Protein Synthesis in a Hyperthermophilic Archaeon[▽]

Thomas J. Santangelo, L'ubomíra Čuboňová,† and John N. Reeve*

Department of Microbiology, Ohio State University, Columbus, Ohio 43210

Shuttle vectors that replicate stably and express selectable phenotypes in both *Thermococcus kodakaraensis* and *Escherichia coli* have been constructed. Plasmid pTN1 from *Thermococcus nautilis* was ligated to the commercial vector pCR2.1-TOPO, and selectable markers were added so that *T. kodakaraensis* transformants could be selected by Δ*trpE* complementation and/or mevinolin resistance. Based on Western blot measurements, shuttle vector expression of RpoL-HA, a hemagglutinin (HA) epitope-tagged subunit of *T. kodakaraensis* RNA polymerase (RNAP), was ~8-fold higher than chromosome expression. An idealized ribosome binding sequence (5′-AGGTGG) was incorporated for RpoL-HA expression, and changes to this sequence reduced expression. Changing the translation initiation codon from AUG to GUG did not reduce RpoL-HA expression, but replacing AUG with UUG dramatically reduced RpoL-HA synthesis. When functioning as translation initiation codons, AUG, GUG, and UUG all directed the incorporation of methionine as the N-terminal residue of RpoL-HA synthesized in *T. kodakaraensis*. Affinity purification confirmed that an HA- plus six-histidine-tagged RpoL subunit (RpoL-HA-his$_6$) synthesized ectopically from a shuttle vector was assembled in vivo into RNAP holoenzymes that were active and could be purified directly from *T. kodakaraensis* cell lysates by Ni^{2+} binding and imidazole elution.

Archaea, in common with *Bacteria*, have small circular genomes not encased in a nuclear compartment, with many genes organized and cotranscribed in operons. Archaeal genome replication and expression machineries, however, have many features more similar to their eukaryotic than to their bacterial counterparts (1, 5, 9, 15). Progress in understanding archaeal replication and gene expression has been made using purified components in vitro, but in vivo validation of the results so obtained has been limited by the lack of genetic systems. This shortcoming has been most pronounced for the thermophilic and hyperthermophilic *Euryarchaea*, many of which are the foci of physiological and biochemical investigations (5, 9). Fortunately progress is now being made, most notably with *Thermococcus kodakaraensis* (2, 8), since it was discovered that *T. kodakaraensis* is naturally competent for DNA uptake and incorporates donor DNA into its genome by homologous recombination (25, 27). Deletion and mutation of chromosomal genes have resulted in the identification of novel biochemical pathways and facilitated the dissection of several events in archaeal transcription in *T. kodakaraensis* (12–14, 17, 19, 22, 23, 26, 28). To overcome the need for homologous recombination, we have now constructed shuttle vectors that replicate and express genes in both *T. kodakaraensis* and *Escherichia coli*. By using plasmid expression, we have documented and quantified the roles of a ribosome binding sequence (RBS) and alternative initiation codons in archaeal translation in *T.

kodakaraensis. We have also established that if one subunit (RpoL) of the multisubunit archaeal DNA-dependent RNA polymerase (RNAP) is synthesized ectopically from a shuttle vector, it is incorporated into functional holoenzymes. Affinity tagging of this plasmid-encoded subunit has then been used to purify the 11-subunit RNAP directly from *T. kodakaraensis* cell lysates.

MATERIALS AND METHODS

Reagents and enzymes. Except where otherwise noted, all chemicals were purchased from Sigma Chemicals (St. Louis, MO); nucleases, restriction enzymes, linear pCR2.1-TOPO plasmid DNA, and TOPO-TA cloning kits were from Invitrogen (Carlsbad, CA); plasmid and PCR product purification kits were from Qiagen Inc. (Valencia, CA); Ni^{2+}-charged HiTrap chelating columns were from GE Healthcare (Piscataway, NJ); and ^{32}P-labeled reagents were from MP Biomedicals (ICN, Irvine, CA).

Media and growth conditions. *T. kodakaraensis* cultures were grown under anaerobic conditions at 85°C in Marine Arts (MA)-yeast extract tryptone (YT) or artificial seawater-amino acids-(AA) medium with or without added tryptophan (50 μg/ml), and cells competent for DNA uptake were prepared as described previously (2, 25). *T. kodakaraensis* KW128 (Δ*pyrF* Δ*trpE::pyrF*) transformants capable of growth in the absence of tryptophan, or the presence of mevinolin, were selected on artificial seawater-AA plates or MA-YT plates containing 15 μM mevinolin, respectively, solidified by incorporation of 1% Gelrite. *E. coli* DH5α cultures were grown in LB medium at 37°C, and transformants were selected on LB plates that contained 100 μg carbenicillin/ml.

Construction of *T. kodakaraensis*-*E. coli* shuttle vectors. A sample of pTN1 was generously provided by N. Soler (29). A preparation of linear pTN1 DNA molecules was generated with 3′-A extensions by PCR amplification using oligonucleotide primers O1 and O2 (5′-TTTTCTGATATTCTGATTTTCTGATA ATTTGCCAATTCTGGCAATTTGGC and 5′-TCAGGAATTTGGAATTTT CAGTTCTAACACATCTGGTGGGTCCTCCCAAG, respectively; Fig. 1A). A pTN1 molecule from this preparation was ligated with pCR2.1-TOPO linearized at position 293, resulting in the entire pTN1 sequence being cloned within, and flanked by, regions of the pCR2.1-TOPO multiple cloning site. The resulting plasmid, designated pTK01, was amplified in *E. coli* DH5α, and both strands were fully sequenced to confirm the construction (Fig. 1A).

An ~1.5-kbp DNA molecule (*trpE* cassette; Fig. 1A) that expresses the *T.

* Corresponding author. Mailing address: Department of Microbiology, Ohio State University, Columbus, OH 43210-1292. Phone: (614) 292-2301. Fax: (614) 292-8120. E-mail: reeve.2@osu.edu.
† Permanent address: Institute of Animal Biochemistry and Genetics, Ivanka Pri Dunayi, Slovak Republic.
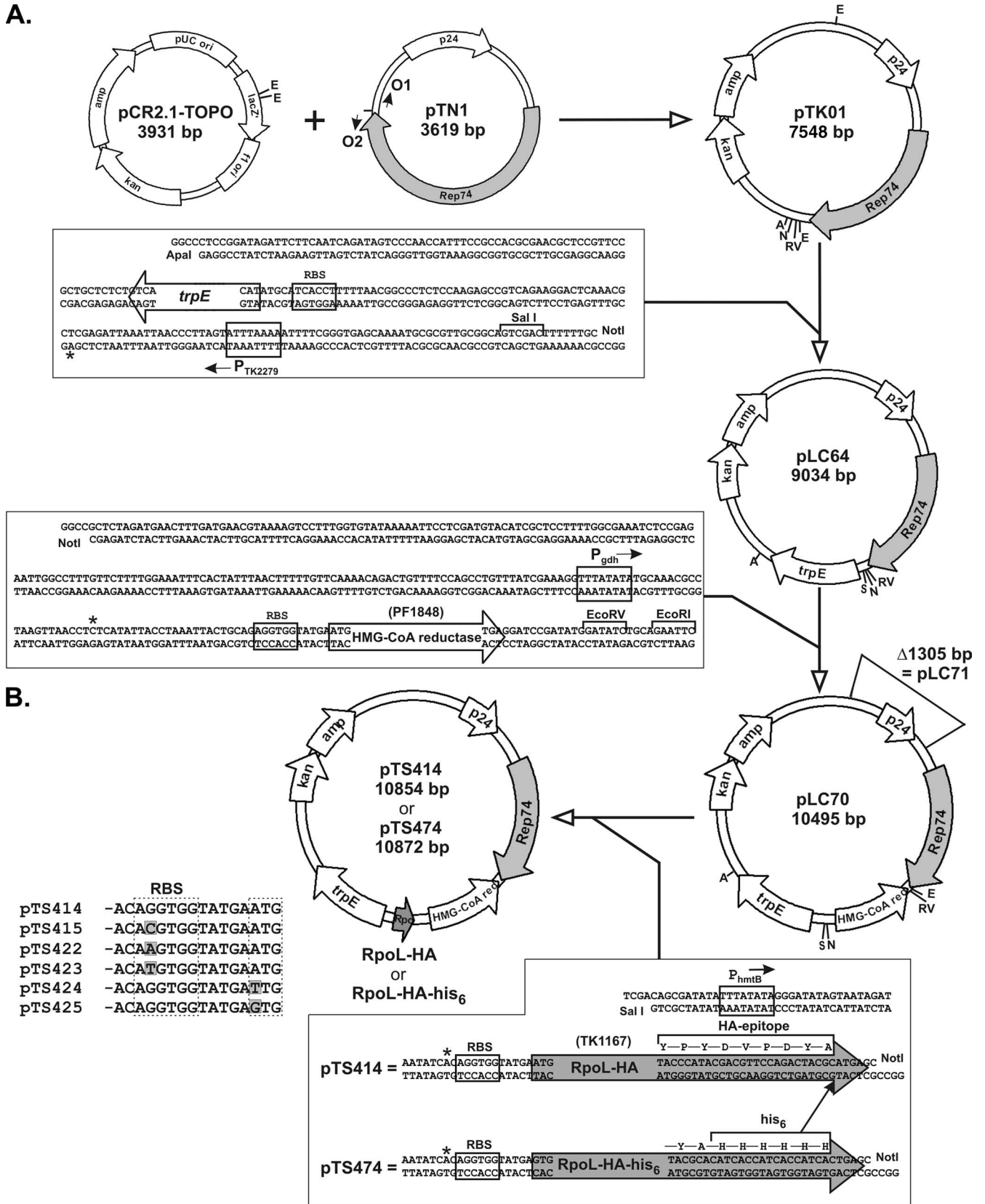
A.

B.

FIG. 1. Shuttle and expression plasmid constructions. (A) The pTN1 sequence (29) is available in GenBank (accession no. EF495263), and the pCR2.1-TOPO sequence is available at http://www.genomex.com/vector_sequence/PCR2.1_Topo.txt. To obtain pTK01, a linear pTN1 DNA molecule, generated by PCR amplification using primers O1 and O2, was ligated with pCR2.1-TOPO linearized at position 293 between the two

kodakaraensis trpE gene constitutively from the *T. kodakaraensis* P_{TK2279} promoter was constructed previously (23). As illustrated in Fig. 1A, this cassette was ligated into pTK01, resulting in plasmid pLC64, and aliquots of pLC64 DNA were used to transform *T. kodakaraensis* KW128 ($\Delta pyrF$ $\Delta trpE$::*pyrF*), with transformants selected by growth on plates in the absence of tryptophan. Cultures of several independent transformants were grown in MA-YT medium, and plasmid preparations were isolated from 3-ml aliquots. The cells were suspended in 0.5 ml of 100 mM Tris-HCl, 100 mM NaCl, 50 mM EDTA (pH 8) that contained 10 μg RNase A and lysed by the addition of 25 μl of 20% (wt/vol) sodium dodecyl sulfate (SDS), mixing, and addition of 20 μl of 5 M NaOH. After 5 min at room temperature, 350 μl of 5 M potassium acetate (pH 4.8) was added and the precipitated material was removed by centrifugation. The supernatant was loaded onto a minipreparation spin column, and bound DNA was washed with 500 μl phosphate buffer, followed by 750 μl phosphatidylethanolamine (Qiagen), and then eluted using 30 to 50 μl of 10 mM Tris-HCl (pH 8). Restriction enzyme digestion and sequencing confirmed that the plasmid isolated from several independent *T. kodakaraensis* prototrophic transformants was pLC64.

Expression of PF1848, a hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase)-encoding gene cloned from *Pyrococcus furiosus*, in *T. kodakaraensis* confers resistance to simvastatin and mevinolin (17, 23). A ~1.5-kbp DNA molecule (Mev^r cassette; Fig. 1A) that has PF1848 transcribed from the constitutive *T. kodakaraensis* P_{gdh} promoter (8) was cloned from pTS429 (23) into pLC64 to generate pLC70 (Fig. 1A). Transformation of *T. kodakaraensis* KW128 with pLC70 resulted in complementation of the $\Delta trpE$::*pyrF* mutation and conferred resistance to >30 μM mevinolin.

Southern blot measurements of plasmid copy number in *T. kodakaraensis*. Aliquots (1 to 7 μg) of total DNA isolated from *T. kodakaraensis* KW128 (pLC70) cells, grown to mid-exponential phase, were digested twice with 20 U of SalI for 90 min at 37°C. The resulting restriction fragments were separated by electrophoresis through 1.1% (wt/vol) agarose gels and then transferred to Zeta-probe membranes (Bio-Rad, Oakland, CA). The membranes were incubated with two hybridization probes (~400 bp), one specific for TK1280, a single-copy chromosomal gene (8, 22), and the other specific for *trpE* (carried on pLC70), ^{32}P labeled by random priming using NEBlot kits (New England Biolabs, Ipswich, MA). The specific activities of the probes were established by direct ^{32}P counting, and dilutions were spotted onto 3MM paper to provide standard curves. These were measured concurrently with the amounts of the ^{32}P-labeled probes that hybridized to the SalI fragment that contained the complementary TK1280 or *trpE* sequence bound to Zeta-probe membrane.

Construction of plasmids that encode RpoL-HA and RpoL-HA-his₆. The gene that encodes RpoL, one of the 11 subunits of the *T. kodakaraensis* RNAP, was previously cloned (22). The coding sequence was extended in frame by ligation of oligonucleotide sequences that added either a hemagglutinin (HA) epitope (YPYDVPDYA; HA tag) or the HA tag plus six histidine residues (HA-his₆) to the C terminus of RpoL (Fig. 1B). These DNA molecules were cloned into pLC70, generating pTS414 and pTS474, respectively. Derivatives of pTS414 were generated with the RBS (pTS415, pTS422, and pTS423) or translation initiation codon (pTS424 and pTS425) changed by using site-specific mutagenic oligonucleotides with QuikChange-XL mutagenesis kits (Stratagene, La Jolla, CA).

Western blot measurements of RpoL-HA synthesis in *T. kodakaraensis*. Cultures of *T. kodakaraensis* KW128, *T. kodakaraensis* TS366 (22), and *T. kodakaraensis* strains containing plasmid pLC70, pTS414, pTS415, pTS422, pTS423, pTS424, or pTS425 were grown in MA-YT-pyruvate medium to mid-exponential phase. The cells were harvested and lysed, and the protein concentrations of the lysates were determined by Bradford assays. Identical quantities of protein from each lysate were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the separated polypeptides were transferred to polyvinylidene difluoride membranes (GE Healthcare). The membranes were incubated, under Western blotting conditions, with anti-HA antibodies (Covance Research Products, Denver, PA), and the complexes formed were visualized by using the ECF system (GE Healthcare) and quantified by using a Storm 840 phosphorimager (GE Healthcare).

Purification of RpoL-HA and in vitro transcription using *T. kodakaraensis* components. The column chromatographic protocol used to purify RNAP and RNAP containing the RpoL-HA subunit from *T. kodakaraensis* and the reagents and conditions used for in vitro transcription using *T. kodakaraensis* components have been described previously (22). The polypeptides in purified RNAP preparations were separated by SDS-PAGE, and the region of the gel that contained RpoL-HA was excised. Preparations of RpoL-HA so purified were fragmented and subjected to mass spectrometry (at Cornell University, Life Sciences Core Laboratories Center, Ithaca, NY) and to N-terminal sequencing by Edman degradation (at the Iowa State University, Biotechnology Services Facility, Ames, IA).

Ni²⁺ affinity purification of RpoL-HA-his₆-tagged RNAP from *T. kodakaraensis*. *T. kodakaraensis* KW128(pLC70) and *T. kodakaraensis* KW128(pTS474) cultures were grown to mid-exponential phase in MA-YT-pyruvate medium containing 5 μM mevinolin (23). The cells were harvested; resuspended in 25 mM Tris-HCl (pH 8), 1 M NaCl, 10% (vol/vol) glycerol; and lysed by freeze-thawing three times using liquid N_2. Particulates were removed by centrifugation, and aliquots of the resulting clarified supernatants were loaded onto 10-ml Ni²⁺-charged HiTrap chelating columns. After being washed with 25 mM Tris-HCl (pH 8), 1 M NaCl, 10% glycerol, material that bound to the column was eluted using a linear gradient of 0 to 500 mM imidazole dissolved in 25 mM Tris-HCl (pH 8), 100 mM NaCl, and 10% glycerol. The polypeptides present in aliquots of the eluted material were separated by SDS-PAGE and visualized by Coomassie blue staining.

RESULTS

***T. kodakaraensis*-*E. coli* shuttle vectors.** Plasmid pTN1 was isolated by Soler et al. (29) from *Thermococcus nautilus*, a relative of *T. kodakaraensis*, and sequenced (3,619 bp), revealing the presence of two long open reading frames designated Rep74 and p24 (29). The Rep74-encoded protein (Rep74; ~74 kDa) has a sequence ~60% homologous to that of the replication protein Rep75 characterized from the *Pyrococcus abyssi* plasmid pGT5 (7, 16). The p24-encoded protein (p24; ~24 kDa) has no clearly recognizable relatives but does bind and compact DNA (29). The entire pTN1 sequence was cloned within the multiple cloning site region of pCR2.1-TOPO. The resulting plasmid, designated pTK01 (Fig. 1A), retains all of the multiple cloning sites, replicates stably, and confers ampicillin and kanamycin resistance on *E. coli*. To provide a selectable phenotype for *T. kodakaraensis* KW128 ($\Delta pyrF$ $\Delta trpE$::*pyrF*) transformation, a *trpE* expression cassette (23) was ligated into pTK01, resulting in pLC64 (Fig. 1A). Transformation of *T. kodakaraensis* KW128 ($\Delta pyrF$ $\Delta trpE$::*pyrF*) with pLC64 resulted in tryptophan-independent growth, and the reisolation of intact pLC64 from representative transformants confirmed that this resulted from plasmid expression of *trpE* complementing the chromosomal $\Delta trpE$::*pyrF* mutation.

To add a second selectable phenotype, a cassette that expresses PF1848 (encoding HMG-CoA reductase) from *P. furiosus* in *T. kodakaraensis* (23) was cloned into pLC64, generating pLC70 (Fig. 1A). Transformation of *T. kodakaraensis* KW128 with pLC70 resulted in transformants that could be selected on the basis of either tryptophan-independent growth

---

EcoRI sites (E), as purchased from Invitrogen. The *trpE*-containing DNA sequence shown was ligated with ApaI (A)- plus NotI (N)-digested pTK01 to produce pLC64. The DNA sequence shown containing the HMG-CoA reductase-encoding gene (PF1848) was ligated with NotI- plus EcoRV (RV)-digested pLC64 DNA to produce pLC70. The asterisks identify the sites of transcription initiation. Deletion of the 1,305-bp region indicated from pLC70 generated pLC71. (B) The DNA sequences shown that encode RpoL-HA and RpoL-HA-his₆ were ligated with SalI (S)- plus NotI-digested pLC70 to generate pTS414 and pTS474, respectively. The RBS regions immediately upstream and the initiation codons of the RpoL-HA-encoding gene in pTS414, pTS415, pTS422, pTS423, pTS424, and pTS425 are shown aligned with the single-nucleotide differences highlighted.

or resistance to mevinolin (Mev[r]). As mevinolin resistance could also be selected on plates containing nutrient-rich medium plus mevinolin, transformation with pLC70 was not limited to *T. kodakaraensis* strains that carry the Δ*trpE*::*pyrF* mutation. Plasmid preparations were purified from several independent *T. kodakaraensis* transformants, and restriction enzyme digestions and sequencing confirmed that, in all cases, the plasmid isolated was intact pLC70. There were no detectable differences in the numbers of *T. kodakaraensis* transformants (~1/10[8] recipient cells) or *E. coli* transformants (~10[6]/10[8] recipient cells) generated when the recipient cells were transformed with 1 μg of pLC70 DNA purified from *T. kodakaraensis* or from *E. coli*.

Shuttle vector stability and copy number in *T. kodakaraensis*. Based on measurements of optical density at 600 nm, cultures of *T. kodakaraensis* KW128 and *T. kodakaraensis* KW128 (pLC70) had identical growth rates in MA-YT-pyruvate medium, and cultures of *T. kodakaraensis* KW128(pLC70) grew at the same rates with or without mevinolin present. To determine if selective pressure were necessary to maintain pLC70 in *T. kodakaraensis* KW128(pLC70), cultures were grown in MA-YT-pyruvate medium without mevinolin, diluted 50-fold, and regrown through seven serial passages. Samples were then diluted and plated on nonselective medium to obtain single colonies. Cells from >80% of the isolated colonies so generated still contained pLC70.

To determine the shuttle vector copy number, Southern blots of SalI-digested total DNA isolated from exponentially growing *T. kodakaraensis* KW128(pLC70) cells were generated and quantified. Based on ^{32}P-labeled probe hybridizations, the SalI restriction fragment that contained *trpE* (from pLC70) was present in these DNA preparations at an ~3-fold-higher abundance than the SalI fragment that carried TK1280 (from the *T. kodakaraensis* chromosome). Given this result, although the number of chromosomes in a growing *T. kodakaraensis* cell remains to be established, pLC70 (and presumably all pTN1-based replicons) is present in such cells at a ratio of ~3 per chromosome.

Replication of pLC70 in *T. kodakaraensis* requires Rep74 but not p24. Derivatives of pLC70 were generated to determine if Rep74 and p24 were essential for pLC70 propagation in *T. kodakaraensis*. As shown in Fig. 1A, an ~1.3-kbp region was deleted from pLC70 that removed the entire p24 coding sequence and a putative downstream ~250-bp insertion sequence (29). The smaller plasmid generated, designated pLC71, replicated stably and conferred tryptophan-independent growth and Mev[r] on *T. kodakaraensis* KW128. In contrast, deleting a single base pair in the Rep74 gene resulted in a plasmid (pLC60) that was stable and replicated in *E. coli* but that could not be propagated in *T. kodakaraensis*. This deletion in codon 487 placed a nonsense codon in frame at position 490, resulting in an open reading frame that encodes a ~56-kDa C-terminally truncated version of Rep74. If this Rep74 variant is synthesized, it does not support plasmid maintenance in *T. kodakaraensis*.

Measurement of RBS and initiation codon contributions to translation in *T. kodakaraensis*. Western blot assays, using anti-HA antibodies, were used to measure and compare the levels of RpoL-HA synthesized in vivo in *T. kodakaraensis* strains. The RpoL-HA-encoding gene was either integrated
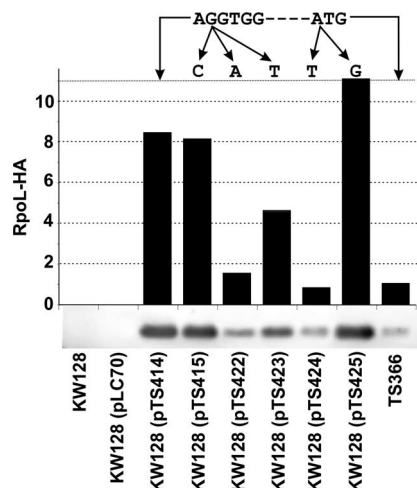

FIG. 2. Synthesis of RpoL-HA in *T. kodakaraensis*. The relative amounts of RpoL-HA present in extracts of *T. kodakaraensis* KW128, *T. kodakaraensis* TS366, and *T. kodakaraensis* KW128 strains containing the plasmids listed. A phosphorimager (Storm 840; GE Healthcare) was used to measure the fluorescence emissions from the Western blot shown generated using anti-HA antibodies. In three independent repetitions of this experiment, the results were essentially identical. The sequence of the RBS and the initiation codon of the RpoL-HA gene integrated into the chromosome of *T. kodakaraensis* TS366 (22) and present in pTS414 (Fig. 1) are provided above the figure with the sequence changes in pTS415 and pTS422 through pTS425 indicated.

into the chromosome (*T. kodakaraensis* TS366 [22]) or expressed from a plasmid (pTS414, pTS415, pTS422, pTS423, pTS424, or pTS425 [Fig. 1B]). As expected, there was no RpoL-HA detectable in lysates of the control cultures, *T. kodakaraensis* KW128 and *T. kodakaraensis* KW128(pLC70), but RpoL-HA was present in the lysates of all cells that contained an RpoL-HA-encoding gene (Fig. 2). Plasmid expression from pTS414 resulted in an ~8-fold-higher abundance of RpoL-HA than did expression from the chromosome in *T. kodakaraensis* TS366 (22). Changing the first G in the RBS to C (pTS415; Fig. 1B) had no detrimental effect on plasmid-directed RpoL-HA synthesis, but replacing the G with A (pTS422) or T (pTS423) substantially reduced RpoL-HA synthesis. Changing the ATG initiation codon to GTG (pTS425) did not decrease, but rather marginally increased, RpoL-HA synthesis, while making TTG the initiation codon dramatically reduced RpoL-HA synthesis (Fig. 2).

RNAP preparations were purified (22) from *T. kodakaraensis* KW128 strains expressing pTS414, pTS424, or pTS425, and the RpoL-HA subunit was gel purified. Both N-terminal amino acid sequencing and mass spectrometry confirmed that, in every case, the N-terminal residue of the RpoL-HA was methionine. Therefore, not only AUG, but also GUG and UUG, directed methionine incorporation into RpoL-HA when functioning as translation initiation codons in *T. kodakaraensis*.

Plasmid expression, holoenzyme assembly, and affinity purification of HA-his[6]-tagged *T. kodakaraensis* RNAP. Purification of multisubunit enzymes intact, as well as the definitive identification of all of the polypeptides that constitute such enzymes in vivo, is often difficult. One approach to these issues is to construct strains that have a known subunit of the enzyme
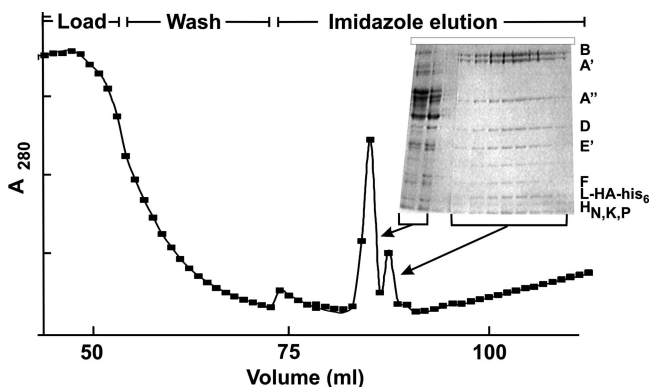
FIG. 3. Purification of RpoL-HA-his$_6$-tagged RNAP. A_{280} measurements were made of fractions obtained from a Ni^{2+}-charged chelating column, loaded with clarified *T. kodakaraensis*(pTS474) cell lysate, washed, and eluted using a 0 to 500 mM imidazole gradient. As shown, the material eluted by imidazole formed two discrete peaks of A_{280} absorbing material. The second peak contained almost exclusively polypeptides with the electrophoretic mobilities established previously (22) for the indicated subunits of purified *T. kodakaraensis* RNAP.

modified to facilitate immunoprecipitation or affinity matrix capture and so purification of the entire enzyme. Plasmid pTS474 was constructed (Fig. 1B) to evaluate this approach with *T. kodakaraensis*. Lysates of *T. kodakaraensis* KW128 (pLC70) and *T. kodakaraensis* KW128(pTS474) were loaded onto Ni^{2+}-charged HiTrap chelating columns and washed, and the bound proteins were then eluted using an imidazole gradient. Imidazole eluted only one peak of A_{280} absorbing material from columns loaded with *T. kodakaraensis* KW128 (pLC70) proteins but eluted two distinct peaks of A_{280} absorbing material from columns bound by proteins from *T. kodakaraensis* KW128(pTS474) (Fig. 3). The second peak contained predominantly 10 polypeptides that migrated as previously established for the subunits of RNAP purified from *T. kodakaraensis* (22), plus RpoL-HA-his$_6$, which migrated slightly slower than RpoL (Fig. 3). RpoL-HA-his$_6$ was therefore assembled into RNAP holoenzymes in vivo, and this facilitated purification of the entire enzyme by affinity to the Ni^{2+}-charged chelating matrix. The RNAP so purified was active in vitro and in in vitro transcription assays generated results (not shown) indistinguishable from those obtained using RNAP preparations that contained RpoL or RpoL-HA purified from *T. kodakaraensis* strains by the established column chromatography protocol (22).

DISCUSSION

We have established that plasmids that contain both the ColE1 and pTN1 replication machineries are maintained stably in both *E. coli* and *T. kodakaraensis*, and by adding genes that provide selectable phenotypes, *E. coli-T. kodakaraensis* shuttle vectors have been constructed (Fig. 1). The minimal region(s) of pTN1 required for replication of these bacterial-archaeal shuttle vectors in *T. kodakaraensis* remains to be defined, but we have established that p24, one of the two proteins known to be encoded by pTN1 (29), is not essential. Rep74 is apparently essential, consistent with its ~60% amino acid sequence homology and likely functional homology with Rep75,

a replication protein encoded by plasmid pGT5 in *P. abyssi* (7, 16). In this regard, it does, however, seem noteworthy that pGT5 has an ~10-fold-higher copy number in *P. abyssi* (16) than we estimate for pLC70 in *T. kodakaraensis*. We have constructed potential shuttle vectors by ligating pGT5 and pCR2.1-TOPO sequences, but none have replicated stably in *T. kodakaraensis*.

Expression of shuttle plasmid-carried genes in *T. kodakaraensis* has been shown to complement a chromosomal deletion (Δ*trpE*) and confer antibiotic (Mev^r) resistance. Plasmid expression has also been used to add a sequence to a multisubunit enzyme (HA- and/or His$_6$ tag) that facilitates Western blot quantification (Fig. 2) and/or affinity purification together with any tightly associated proteins (Fig. 3). Plasmid overexpression should also facilitate more-rapid purification of any enzyme of interest directly from *T. kodakaraensis*. Furthermore, by providing the necessary complementing activity from a plasmid, it should now be possible to mutate and manipulate essential genes in the *T. kodakaraensis* genome. Ideally, complementation would be controlled by using a promoter that can be regulated by the addition or removal of a gratuitous reagent, and several candidate promoter, regulator, and reagent combinations have been evaluated but, to date, without success. As an alternative, we have established that very different concentrations of a plasmid-encoded protein can be logically obtained in vivo in *T. kodakaraensis* by manipulating the RBS and/or changing the initiation codon (Fig. 2).

Based on the translation factors encoded in archaeal genomes, in vitro structural and functional studies, and bioinformatics scrutiny of sequences (1, 3, 4, 6, 8, 11, 15, 18, 21, 24, 31, 33), archaeal translation initiation has both bacterial and eukaryotic features. Almost all studies of archaeal translation in vitro have employed components isolated from *Sulfolobus solfataricus*, and the results obtained argue that translation initiation in this species occurs by two mechanisms, one dependent on and one independent of an RBS (3, 6, 11, 15). Several examples of transcription initiation occurring so close to the translation initiation codon that the resulting mRNA cannot contain an upstream RBS support extrapolating this conclusion to other *Archaea* (1, 4, 15, 30, 31). To our knowledge, only two previous reports describe the results of in vivo investigations of archaeal translation initiation (24, 30), both of which took advantage of the availability of a transformation system for the mesophilic halophile *Halobacterium salinarum*. As documented here for the RBS variants in *T. kodakaraensis*, changes introduced into a sequence predicted to be a natural RBS did result in differences in the levels of protein synthesis in vivo in *H. salinarum* (24). The frequent use of GTG and TTG as initiation codons in *Archaea* resulted initially in many genome misannotations (20, 31, 32), but with this now well recognized, establishing how different initiation codons contribute to translation in *Archaea* is clearly of experimental interest (30). As predicted by structural studies (1, 11, 18, 33) and consistent with most (32) but not all (10) previous reports, all three initiation codons directed incorporation of methionyl in *T. kodakaraensis* as the N-terminal residue of RpoL-HA. The results obtained also argue that UUG directs translation initiation much less efficiently in *T. kodakaraensis* than does AUG or GUG, at least when substituted into a transcript in which AUG is the natural initiation codon.

REFERENCES

1. **Allen, G. S., and J. Frank.** 2007. Structural insights on the translation initiation complex: ghosts of a universal initiation complex. Mol. Microbiol. **63:**941–950.
2. **Atomi, H., T. Fukui, T. Kanai, M. Morikawa, and T. Imanaka.** 2004. Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. Archaea **1:**263–267.
3. **Benelli, D., E. Maone, and P. Londei.** 2003. Two different mechanisms for ribosome/mRNA interaction in archaeal translation initiation. Mol. Microbiol. **50:**635–643.
4. **Brennels, M., O. Hering, C. Lange, and J. Soppa.** 2007. Experimental characterization of *cis*-acting elements important for translation and transcription in halophilic *Archaea*. PLoS Genet. **3:**2450–2467.
5. **Cavicchioli, R.** 2007. Archaea. Molecular and cellular biology. ASM Press, Washington, DC.
6. **Condò, I., A. Ciammaruconi, D. Benelli, D. Ruggero, and P. Londei.** 1999. *cis*-acting signals controlling translational initiation in the thermophilic archaeon *Sulfolobus solfataricus*. Mol. Microbiol. **34:**377–384.
7. **Erauso, G., S. Marsin, N. Benbouzid-Rollet, M. F. Baucher, T. Barbeyron, Y. Zivanovic, D. Prieur, and P. Forterre.** 1996. Sequence of plasmid pGT5 from the archaeon *Pyrococcus abyssi*: evidence for rolling-circle replication in a hyperthermophile. J. Bacteriol. **178:**3232–3237.
8. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. Genome Res. **15:**352–363.
9. **Garrett, R. A., and H.-P. Klenk.** 2007. Archaea. Evolution, physiology, and molecular biology. Blackwell Publishing, Oxford, United Kingdom.
10. **Hansen, T., B. Reichstein, R. Schmid, and P. Schönheit.** 2002. The first archaeal ATP-dependent glucokinase, from the hyperthermophilic crenarchaeon *Aeropyrum pernix*, represents a monomeric, extremely thermostable ROK glucokinase with broad hexose specificity. J. Bacteriol. **184:**5955–5965.
11. **Hasenöhrl, D., D. Benelli, A. Barbazza, P. Londei, and U. Bläsi.** 2006. *Sulfolobus solfataricus* translation initiation factor 1 stimulates translation initiation complex formation. RNA **12:**674–682.
12. **Hu, G.-Q., X. Zheng, Y.-F. Yang, P. Ortet, Z.-S. She, and H. Zhu.** 2008. ProTISA: a comprehensive resource for translation initiation site annotation in prokaryotic genomes. Nucleic Acids Res. **36:**D114–D119.
13. **Imanaka, H., A. Yamatsu, T. Fukui, H. Atomi, and T. Imanaka.** 2006. Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden-Meyerhof pathway in *Thermococcus kodakaraensis*. Mol. Microbiol. **61:**898–909.
14. **Kanai, T., J. Akerboom, S. Takedomi, H. J. van de Werken, F. Blombach, J. van der Oost, T. Murakami, H. Atomi, and T. Imanaka.** 2007. A global transcriptional regulator in *Thermococcus kodakaraensis* controls the expression levels of both glycolytic and gluconeogenic enzyme-encoding genes. J. Biol. Chem. **282:**33659–33670.
15. **Londei, P.** 2005. Evolution of translation initiation: new insights from the archaea. FEMS Microbiol. Rev. **29:**185–200.
16. **Lucas, S., L. Toffin, Y. Zivanovic, D. Charlier, H. Moussard, P. Forterre, D. Prieur, and G. Erauso.** 2002. Construction of a shuttle vector for, and spheroplast transformation of, the hyperthermophilic archaeon *Pyrococcus abyssi*. Appl. Environ. Microbiol. **68:**5528–5536.
17. **Matsumi, R., K. Manabe, T. Fukui, H. Atomi, and T. Imanaka.** 2007. Disruption of a sugar transporter gene cluster in a hyperthermophilic archaeon using a host-marker system based on antibiotic resistance. J. Bacteriol. **189:**2683–2691.
18. **Nikonov, O., E. Stolboushkina, A. Nikulin, D. Hasenöhrl, U. Bläsi, D. Manstein, R. Fedorov, M. Garber, and S. Nikonov.** 2007. New insights into the interactions of the translation initiation factor 2 from *Archaea* with guanine nucleotides and initiator tRNA. J. Mol. Biol. **373:**328–336.
19. **Orita, I., T. Sato, H. Yurimoto, N. Kato, H. Atomi, T. Imanaka, and Y. Sakai.** 2006. The ribulose monophosphate pathway substitutes for the missing pentose phosphate pathway in the archaeon *Thermococcus kodakaraensis*. J. Bacteriol. **188:**4698–4704.
20. **Poole, F. L., B. A. Gerwe, R. C. Hopkins, G. J. Schut, M. V. Weinberg, F. E. Jenney, Jr., and M. W. W. Adams.** 2005. Defining genes in the genome of the hyperthermophilic archaeon *Pyrococcus furiosus*: implications for all microbial genomes. J. Bacteriol. **187:**7325–7332.
21. **Ring, G., P. Londei, and J. Eichler.** 2007. Protein biogenesis in *Archaea*: addressing translation initiation using an in vitro protein synthesis system for *Haloferax volcanii*. FEMS Microbiol. Lett. **270:**34–41.
22. **Santangelo, T. J., L. Čuboňová, C. L. James, and J. N. Reeve.** 2007. TFB1 or TFB2 is sufficient for *Thermococcus kodakaraensis* viability and for basal transcription *in vitro*. J. Mol. Biol. **367:**344–357.
23. **Santangelo, T. J., L. Čuboňová, R. Matsumi, H. Atomi, T. Imanaka, and J. N. Reeve.** 2008. Polarity in archaeal operon transcription in *Thermococcus kodakaraensis*. J. Bacteriol. **190:**2244–2248.
24. **Sartorius-Neef, S., and F. Pfeifer.** 2004. In vivo studies on putative Shine-Dalgarno sequences of the halophilic archaeon *Halobacterium salinarum*. Mol. Microbiol. **51:**579–588.
25. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. J. Bacteriol. **185:**210–220.
26. **Sato, T., H. Imanaka, N. Rashid, T. Fukui, H. Atomi, and T. Imanaka.** 2004. Genetic evidence identifying the true gluconeogenic fructose-1,6-bisphosphatase in *Thermococcus kodakaraensis* and other hyperthermophiles. J. Bacteriol. **186:**5799–5807.
27. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2005. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. Appl. Environ. Microbiol. **71:**3889–3899.
28. **Sato, T., H. Atomi, and T. Imanaka.** 2007. Archaeal type III RuBisCOs function in a pathway for AMP metabolism. Science **315:**1003–1006.
29. **Soler, N., A. Justome, S. Quevillion-Cheruel, F. Lorieux, E. LeCam, E. Marguet, and P. Forterre.** 2007. The rolling-circle plasmid pTN1 from the hyperthermophilic archaeon *Thermococcus nautilus*. Mol. Microbiol. **66:**357–370.
30. **Srinivasan, G., M. P. Krebs, and U. L. RajBhandary.** 2006. Translation initiation with GUC codon in the archaeon *Halobacterium salinarum*: implications for translation of leaderless mRNA and strict correlation between translation initiation and presence of mRNA. Mol. Microbiol. **59:**1013–1024.
31. **Torarinsson, E., H.-P. Klenk, and R. A. Garrett.** 2005. Divergent transcriptional and translational signals in *Archaea*. Environ. Microbiol. **7:**47–54.
32. **Yamazaki, S., J. Yamazaki, K. Nishijima, R. Otsuka, M. Mise, H. Ishikawa, K. Sasaki, S.-I. Tago, and K. Isono.** 2006. Proteome analysis of an aerobic hyperthermophilic crenarchaeon, *Aeropyrum pernix* K1. Mol. Cell. Proteomics **5:**811–823.
33. **Yatime, L., E. Schmitt, S. Blanquet, and Y. Mechulam.** 2004. Functional molecular mapping of archaeal initiation factor 2. J. Biol. Chem. **279:**15984–15993.