Polarity in Archaeal Operon Transcription in *Thermococcus kodakaraensis*[▽]

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An in vivo archaeal gene reporter system has been established based on TK1761, a gene that encodes a nonessential β -glycosidase in *Thermococcus kodakaraensis*. Following the introduction of nonsense codons into promoter-proximal genes, polarity in operon expression in this archaeon has been established by both microarray hybridization assays and a reporter gene expression system.

Archaea lack a nuclear membrane, and so ribosomes can bind and initiate translation before a transcript is complete (8). In *Bacteria*, such immediate translation of nascent 5' sequences often signals transcription to terminate at attenuators upstream of polycistronic operons (12, 19). The close association of the bacterial translation and transcription machineries also prevents premature transcription termination during operon transcription (1, 3, 5, 20). If translation is halted by a nonsense codon, transcription without translation generates an exposed transcript to which the transcription termination factor rho (ρ) can bind. Rho then translocates along the RNA, catches and disrupts the transcription complex, and terminates transcription, reducing the expression of all genes downstream of the nonsense codon; this phenomenon is known as nonsense-codon-mediated polarity (3, 5, 16, 20). *Archaea* also have many genes organized and cotranscribed within multigene operons, and given the many systems present in *Bacteria* that exploit transcription and translation coupling to regulate operon expression (3, 19), it seems likely that such systems should also have evolved in *Archaea*. However, archaeal genome sequences provide no evidence for attenuators, and they do not encode homologues of ρ . There is also no experimental support for regulation based on coupling of transcription and translation, and there is only limited complementation evidence for polarity in archaeal operon expression (6, 15, 21, 23).

This lack of information reflects, to a large extent, the difficulties encountered in establishing genetics with *Archaea* and, thus, the inability to probe and manipulate archaeal gene expression in vivo. Many basic features of archaeal molecular biology have been established through in vitro studies (4, 10), but establishing whether translation regulates transcription termination requires substantially more sophisticated in vitro technology than is currently available. Fortunately, with the discovery that *Thermococcus kodakaraensis* (2) is naturally competent for DNA uptake and incorporates donor DNA into its genome by homologous recombination, in vivo approaches are now becoming possible with this hyperthermophilic archaeon (18, 28, 30). By using targeted mutagenesis, progress

has already been made in discovering and dissecting novel archaeal biochemical pathways and in regulation of transcription initiation (13, 14, 18, 22, 27, 29, 31). An important additional tool needed for in vivo investigations is a reporter gene whose expression can be monitored to confirm and quantify the activities of regulatory elements in vivo. Reverse genetics previously established that TK1761 encodes a β -glycosidase (7), and here we report the development of a TK1761-based reporter system for gene expression in *T. kodakaraensis*. This reporter is used to document that nonsense-codon-mediated polarity does occur in operon expression in *T. kodakaraensis*.

Construction of a TK1761-based reporter and expression cassette. *T. kodakaraensis* KW128 (Δ pyrF; Δ trpE::pyrF) is a tryptophan auxotroph, and transformation with donor DNAs that carry the *T. kodakaraensis* *trpE* gene results in prototrophic transformants that grow on minimal medium-containing plates without tryptophan (28, 30). Plasmid pUMT2 was constructed previously with the *T. kodakaraensis* *trpE* gene cloned into the HincII site of pUC118 (28, 29), and plasmid pTS372 was generated from pUMT2 (Fig. 1A) by inserting ~2 kbp of *T. kodakaraensis* genomic DNA both downstream and upstream of the *trpE* gene in pUMT2. After PCR amplification from the *T. kodakaraensis* genome, the intergenic region between TK1760 and TK1761 followed by the TK1760 coding sequence was positioned downstream of *trpE*. Positioned upstream from *trpE* was the TK1761 coding sequence and a 5' fragment of TK1762 (Fig. 1A). A sequence that was generated and cloned between *trpE* and TK1761 contains two divergent promoters, P_{TK2279} from *T. kodakaraensis* and P_{hmtB} from *Methanothermobacter thermautotrophicus*, plus ribosome binding sites (5'-AGGTGA) positioned upstream of both genes (Fig. 1A). Previous in vitro studies established that the *T. kodakaraensis* transcription machinery recognizes and uses P_{hmtB} as a strong promoter (27). This heterologous promoter was used to direct TK1761 expression in vivo to minimize the likelihood of promoter-level regulation in *T. kodakaraensis*. Plasmids pTS416 and pTS419 were generated from pTS372 by using mutagenic oligonucleotides with QuikChange XL mutagenesis kits (Stratagene) that changed the GAG codon at position 3 in TK1761 to TAG and the TATA box sequence of P_{hmtB} from TTTATATA to GGGGATA (Fig. 1A). Aliquots (1 μ g) of pTS372, pTS416, and pTS419 DNAs, purified after amplification in *Escherichia coli* DH5 α , were used to transform

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[▽] Published ahead of print on 11 January 2008.

FIG. 1. Construction and assays of β -glycosidase reporter strains and demonstration of polarity by microarray hybridization. (A). Plasmid pTS372 was derived from pUMT2 (28, 29) by the insertion of *T. kodakaraensis* chromosomal DNA. TK1761 was positioned upstream and TK1760 downstream of *trpE*. A sequence (oval inset) was incorporated between *trpE* and TK1761 in pTS372 that positioned the divergent P_{TK2279} and P_{hmtB} promoters as indicated. The sequence differences in pTS416 and pTS419 are shown above the pTS372 sequence. TATA box promoter and ribosome binding sequences are boxed, and arrows identify the sites of transcription initiation. The presence of the nonsense codon and the inactivation of P_{hmtB} in *T. kodakaraensis* TS416 and TS419 are indicated by the STOP symbol and the X through the transcription initiation arrow, respectively. (B) β -Glycosidase activities present in aliquots of lysates of *T. kodakaraensis* KW128, TS372, TS416, and TS419 assayed by hydrolysis of ONPgluco, ONPmanno, and ONPgalacto. Three separate cultures of each strain were grown, and each lysate was assayed in triplicate. The results shown are the mean values so obtained. (C). Heavy lines indicate the regions of TK1761, TK1762, and TK1763 that were amplified and spotted on the *T. kodakaraensis* microarrays as targets for hybridization. The table lists the differences and percentage reductions in the TK1761, TK1762, and TK1763 transcript abundances in RNA preparations from *T. kodakaraensis* TS416 and TS372.

T. kodakaraensis KW128, and prototrophic transformants were selected by growth on Gelrite solidified minimal medium incubated at 85°C, as previously described (27–29). Cultures of representative transformants were grown to stationary phase in MA-YT medium that contained 5 g sodium pyruvate/liter, the cells were harvested, genomic DNA was isolated (27), and the TK1760-*trpE*-TK1761 region was amplified by PCR. Both strands of the amplified DNAs were sequenced to confirm the location and presence of the desired sequence in the genome of the transformant. Cultures of *T. kodakaraensis* KW128 and three transformants, designated *T. kodakaraensis* TS372,

TS416, and TS419, were grown to mid-exponential phase in MA-YT-pyruvate medium. Cells from 10-ml aliquots were harvested by centrifugation, resuspended in 0.2 ml of 10 mM Tris-HCl (pH 8), and lysed by freezing and thawing three times, using liquid N_2 . Cellular debris was removed by centrifugation ($18,000 \times g$ for 15 min), and the protein concentration of the resulting clarified lysate was determined by Bradford assays. The β -glycosidase activity present in each lysate was determined by monitoring the change in absorbance at 405 nm (A_{405}) during incubation at 85°C after the addition of 2.8 mM ortho-nitrophenyl- β -D-glucopyranoside (ONPgluco), ortho-

nitrophenyl- β -D-mannopyranoside (ONPmanno), or ortho-nitrophenyl- β -D-galactopyranoside (ONPgalacto) dissolved in 50 mM sodium phosphate (pH 6.5). Linear increases in the A_{405} were observed for at least 30 min, and 1 U of activity was defined as catalyzing a ΔA_{405} of 1 in one min/pg of protein.

The protein (pTK1761) encoded by TK1761 was known to have β -glycosidase activity (7), but assays of *T. kodakaraensis* KW128 cell extracts revealed the presence of two chromatographically separable β -glycosidase activities. Comparisons of the activities present in *T. kodakaraensis* KW128 and TS372 lysates confirmed that *T. kodakaraensis* TS372 cells contained substantially higher concentrations of an activity that hydrolyzed ONPgluco and ONPmanno but not ONPgalacto (Fig. 1B). Assays of the activities present in lysates of *T. kodakaraensis* TS416 and TS419 confirmed that the β -glycosidase with this substrate selectivity was encoded by TK1761 and that its synthesis was dependent on an intact P_{hmtB} . The residual β -glycosidase activity in lysates of *T. kodakaraensis* TS416 and TS419 was essentially the same as that in lysates of the parental strain *T. kodakaraensis* KW128 (Fig. 1B). Manipulating the intergenic region upstream of TK1761 therefore had no effect on the expression of the gene that encodes the ONPgalacto hydrolyzing β -glycosidase. Measurements of ONPgluco or ONPmanno hydrolyzing activity, encoded by TK1761, could therefore be used to report and quantify the activities of regulatory elements that direct TK1761 expression in *T. kodakaraensis*.

Demonstration of operon polarity by microarray hybridizations. Construction of *T. kodakaraensis* TS416 also offered an opportunity to investigate if the presence of a nonsense codon in a promoter-proximal gene (TK1761) resulted in decreased transcription of genes downstream in the same operon (TK1762 and TK1763) (Fig. 1C). The TK1762 and TK1763 gene products are annotated as a hypothetical protein and a putative DNA binding protein (9), respectively, but with no defined activities, and so transcript levels were measured directly. RNA preparations from *T. kodakaraensis* TS372 and TS416 cells, harvested at the same density from cultures growing exponentially in MA-YT-pyruvate medium, were reverse transcribed to generate fluorescently labeled cDNA preparations as previously described (14, 29). Hybridization with *T. kodakaraensis* whole-genome microarrays provided six separate comparisons of the abundance of every transcript present in RNA preparations from *T. kodakaraensis* TS372 and TS416. Almost all of the 2,258 transcripts compared were present at essentially the same abundance in RNA preparations from both strains. In contrast, the TK1761, TK1762, and TK1763 transcripts were, respectively, ~ 8 -, 19.5- and 24.6-fold more abundant (mean values) in the RNA preparations from *T. kodakaraensis* TS372 than those from TS416. The presence of the stop codon in TK1761 apparently therefore reduced TK1761, TK1762, and TK1763 transcription by $\sim 87\%$, 95%, and 96%, respectively, in *T. kodakaraensis* TS416 (Fig. 1C), consistent with nonsense-codon-mediated polarity in operon expression.

Demonstration of operon polarity by reporter gene expression. To confirm and further investigate polarity in operon expression in *T. kodakaraensis*, six strains were constructed (designated *T. kodakaraensis* TS429 and TS431 through TS435) that had the TK1761 reporter gene located at the

second position in an operon (Fig. 2). The promoter-proximal gene PF1848 was PCR amplified from *Pyrococcus furiosus* genomic DNA and cloned between P_{hmtB} and TK1761 in pTS372 to obtain pTS429 (Fig. 2). PF1848 encodes hydroxymethylglutaryl-coenzyme A reductase, and the expression of PF1848 confers simvastatin resistance on *T. kodakaraensis* (18). The transformation of *T. kodakaraensis* KW128 with pTS429 DNA resulted in prototrophic transformants that were also resistant to mevinolin, a close relative of simvastatin. PCR amplification and sequencing confirmed the presence of the desired TK1760-*trpE*-PF1848-TK1761 gene organization in the genome of a representative transformant, designated *T. kodakaraensis* TS429. Site-specific oligonucleotide-directed mutagenesis was used to place nonsense codons at five different locations in PF1848 in pTS429, resulting in plasmids pTS431 to pTS435. Aliquots of these plasmid DNAs were used to transform *T. kodakaraensis* KW128, and genomic DNA was prepared from representative prototrophic transformants, designated *T. kodakaraensis* TS431 to TS435 (Fig. 2). For every strain, PCR amplification and sequencing confirmed the presence of the TK1760-*trpE*-PF1848-TK1761 gene organization in the *T. kodakaraensis* genome with the desired mutation in the PF1848 sequence. Cells were harvested from cultures of each strain grown to mid-exponential phase, concentrated, and lysed, and the ONPgluco hydrolyzing activity present in each lysate was measured. As shown in Fig. 2, the β -glycosidase activity was lower in the lysates of all of the strains that had a mutation in PF1848 than the activity in the otherwise isogenic strain, *T. kodakaraensis* TS429. The reductions in β -glycosidase activity resulting from the presence of a nonsense codon at positions 35, 132, 243, 348, and 381 in PF1848 were $\sim 65\%$, 28%, 13%, 39%, and 8%, respectively. These decreases are consistent with the distance between the translation stop codon and the ATG initiation codon of TK1761 playing a role in the extent of polarity, but as this is not an exact correlation, additional parameters apparently contribute to this polarity in *T. kodakaraensis*.

Conclusions. The natural competency of *T. kodakaraensis* (28) has made genetic approaches to investigating the biology and molecular biology of this hyperthermophilic euryarchaeon possible. As an additional tool, we have now established TK1761 as a reporter gene that can be used to detect and quantify the activities of gene regulatory elements in vivo in *T. kodakaraensis*. TK1761 encodes a dispensable β -glycosidase that hydrolyzes ONPgluco and ONPmanno, but not ONPgalacto (Fig. 1B) (7). Either of the two colorimetric substrates can therefore be used to assay pTK1761 activity in cell extracts and, thus, to measure TK1761 expression in vivo from different regulatory signals, under different growth conditions, and in different *T. kodakaraensis* genetic backgrounds.

Transcription and translation are coupled in *T. kodakaraensis* (8) and most likely in all *Archaea*, given that the absence of a nuclear membrane allows ribosomes immediate access to nascent transcripts. Such coupling of transcription and translation is widely exploited for regulation in *Bacteria* (5, 12, 19), and here, for the first time, we demonstrate that uncoupling translation from transcription has a negative effect on downstream transcription and, thus, on operon expression in an archaeon. Placing a nonsense codon in an upstream gene reduced downstream gene expression in vivo when measured

FIG. 2. Construction and reporter assays of *T. kodakaraensis* strains with TK1761 positioned downstream from PF1848. Plasmid pTS429 was generated from pTS372 (Fig. 1A) by replacing a PstI-SphI fragment that contained *P_{hmtB}*, TK1761, and a 5' region of TK1762 with a PstI-SphI fragment that contained *P_{hmtB}*, PF1848, TK1761, and the same 5' region of TK1762. Site-directed mutagenesis was used to place stop codons in PF1848 at the sites indicated by the STOP symbols. The precise location of the nonsense codon and its distance from the ATG start codon of TK1761 in each derivative of pTS429 are listed in the table. Plasmid DNA was used to transform *T. kodakaraensis* KW128, resulting in *T. kodakaraensis* strains TS429 and TS431 through TS435. The β -glycosidase activities present in lysates of these strains, expressed as percentages of the activity present in lysates of *T. kodakaraensis* TS429, are listed. The values are the averages, with errors, for assays of lysates from three separate cultures, with each assayed in triplicate.

both at the level of transcript abundance (Fig. 1) and as gene product activity (Fig. 2). In *Bacteria*, such nonsense-codon-mediated polarity is dependent on ρ (1, 11, 16, 17, 24), and ρ -dependent termination appears to occur downstream of ~50% of the transcription units in *E. coli* (25). The demonstration of polarity in *T. kodakaraensis* raises the question of whether this depends on an archaeal functional homologue of ρ and, if so, whether such a protein also directs transcription termination downstream of genes in *Archaea*. Preliminary studies do suggest that *T. kodakaraensis* contains a protein factor that can disrupt transcription elongation complexes in vitro (T. J. Santangelo, unpublished results), although there is no recognizable homologue of ρ or of any other known bacterial or eukaryotic transcription termination factor encoded in the *T. kodakaraensis* genome (9). Based on the sequences that have been shown to direct intrinsic termination of *T. kodakaraensis* RNA polymerase in vitro (26), very few intergenic regions in the *T. kodakaraensis* genome contain clearly recognizable intrinsic terminators. This finding adds to the likeli-

hood that protein factor-dependent transcription termination does occur in *T. kodakaraensis*, and based on the results in Fig. 2, this factor requires <100 nucleotides of exposed transcript to stimulate termination. If factor-dependent termination occurs in *Archaea*, then two additional questions are immediately raised: how do long noncoding rRNA transcripts avoid termination in *Archaea*, and are there also archaeal antitermination factors (20)?

At Ohio State University, this work was supported by research grants from the Department of Energy (DE-FG02-87ER13731) and National Institutes of Health (GM53185) to J.N.R. and a National Institutes of Health fellowship (1F32-GM073336-01) to T.J.S. At Kyoto University, this work was supported by the Japan Society for the Promotion of Science under a grant-in-aid for Creative Scientific Research (project no. 18GS0421) to T.I. and a grant-in-aid for Scientific Research on Priority Areas "Applied Genomics" (no. 1801026) to H.A. from the Ministry of Education, Culture, Sports and Technology of Japan.

We thank F. Robb for the gift of *P. furiosus* genomic DNA and K. Skinner for technical assistance.

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