RNA (2002), *8*:1129–1136. Cambridge University Press. Printed in the USA.
Copyright © 2002 RNA Society.
DOI: 10.1017.S1355838202021052

Stability of mRNA in the hyperthermophilic archaeon *Sulfolobus solfataricus*

ELISABETTA BINI, VIDULA DIKSHIT, KRISTI DIRKSEN, MELISSA DROZDA, and PAUL BLUM

George Beadle Center for Genetics, University of Nebraska–Lincoln, Lincoln, Nebraska 68588-0666, USA

ABSTRACT

Archaea-like bacteria are prokaryotes but, in contrast, use eukaryotic-like systems for key aspects of DNA, RNA, and protein metabolism. mRNA is typically unstable in bacteria and stable in eukaryotes, but little information is available about mRNA half-lives in archaea. Because archaea are generally insensitive to antibiotics, examination of mRNA stability in the hyperthermophile, *Sulfolobus solfataricus*, required the identification of transcription inhibitors for half-life determinations. An improved *lacS* promoter-dependent in vitro transcription system was used to assess inhibitor action. Efficient inhibitors were distinguished as blocking both *lacSp* transcription in vitro and the incorporation of ^{3}H-uracil into bulk RNA in vivo. Actinomycin D was the most stable and potent compound identified. A survey of transcript chemical half-lives normalized to levels of the signal recognition particle 7S RNA ranged from at least 2 h for *tfb1*, a transcription factor TFIIB paralog, to a minimum of 6.3 min for *gln1*, one of three glutamine synthetase paralogs. Transcript half-lives for other mRNAs were: 2 h, superoxide dismutase (*sod*); 37.5 min, glucose dehydrogenase (*dhg1*); 25 min, alpha-glucosidase (*malA*); and 13.5 min, transcription factor TFIIB-2 (*tfb2*) resulting in a minimum average half-life of 54 min. These are the first mRNA half-lives reported for a hyperthermophile or member of the crenarchaea. The unexpected stability of several transcripts has important implications for gene expression and mRNA degradation in this organism.

Keywords: archaea; hyperthermophiles; mRNA decay; transcription inhibitors

INTRODUCTION

The turnover of mRNA provides a mechanism for readjusting gene expression. In bacterial prokaryotes, the average mRNA half-life ranges from seconds to minutes (Nilsson et al., 1984; Coburn & Mackie, 1999; Grunberg-Manago, 1999) though mRNAs of greater stability also exist (Arnold et al., 1998; Hambraeus et al., 2000). In eukaryotes, average mRNA half-lives are longer, varying from tens of minutes in yeast to hours in mammalian cells (Singer & Penman, 1973; Warner & Gorenstein, 1978; Wang et al., 2002). These differences in mRNA stabilities are a reflection of sequence-dependent structure and the dissimilar occurrence of enzymes for mRNA degradation that are abundant in bacteria and rare in eukaryotes. In bacteria, gene expression strategies are dominated by mechanisms acting at the level of transcription initiation. In eukaryotes, posttranscriptional mechanisms are more widespread.

Reprint requests to: Dr. Paul Blum, E234 George Beadle Center for Genetics, University of Nebraska, Lincoln, Nebraska 68588-0666, USA; e-mail: pblum@biocomp.unl.edu.

The decay of mRNA in *Escherichia coli* is initiated by endonucleolytic cleavage followed by processive digestion in a 3′ to 5′ direction. Only a subset of the many RNases identified in *E. coli* catalyze mRNA degradation. The endoribonucleases include RNAses E, G, and III, whereas the exoribonucleases include RNase II, polynucleotide phosphorylase (PNPase), and oligoribonuclease (OligoRNase; reviewed in Steege, 2000). RNase E, PNPase, and an RNA helicase (RhlB) are associated in a multiprotein complex called the degradosome (Blum et al., 1997; Vanzo et al., 1998). Though no 5′-3′ exonuclease has been detected, the net degradation of mRNA in bacteria occurs in this direction. Short polyadenylate tails are present in certain bacterial mRNAs (reviewed in Sarkar, 1997) that decrease mRNA stability, the opposite of what occurs in eukaryotes (Xu et al., 1993; O'Hara et al., 1995).

Eukaryotic mRNAs possess two unique posttranscriptional modifications, the 5′ methylated guanosine cap and a long 3′ polyadenylate tail that provide barriers to mRNA degradation (reviewed in Ross, 1995; Tharun & Parker, 1997; Mitchell & Tollervey, 2000). Eukaryotic enzymes responsible for stable RNA processing and

degradation as well as some mRNA turnover are localized within a multisubunit complex termed the exosome. However, the major mRNA decay pathway and corresponding activities are not exosome associated, and in *Saccharomyces cerevisiae*, consist of deadenylation (PAN) followed by decapping (DCP1) and subsequent 5′-3′ exonucleolytic (XRN1) degradation. The 3′ untranslated regions can encode *cis*-acting sequences that control transcript half-life. These have the ability to form secondary structures and alter mRNA stability through effects on translation (Shyu et al., 1991; Xu et al., 1997) or by recruiting factors whose binding increases transcript half-life (Seiser et al., 1995).

Archaea constitute the other major group of prokaryotes (Woese & Fox, 1977). They are subdivided into two groups, the crenarchaea, comprising mostly hyperthermophilic organism, and the euryarchaea, which includes a range of biotypes including methanogens and halophiles. The lack of transcription inhibitors has limited efforts to examine mRNA stability in archaea. Though rifampicin has been used extensively in bacteria for mRNA stability measurements, the archaeal RNA polymerase is insensitive to this compound (Zillig et al., 1979). Like bacteria, archaeal mRNAs are not capped, and at least some mRNAs undergo limited polyadenylation (Brown & Reeve, 1985, 1986; Volkl et al., 1996). Measurements of mRNA stability are available only for *Methanococcus vannielii*, a mesophilic (growth at ambient temperatures) euryarchaeote, and ranged from 7 to 57 min (Hennigan & Reeve, 1994). Inhibition of RNA synthesis, however, depended on the use of energy (hydrogen) deprivation and Methyl Co-M Reductase inhibition rather than specific transcription inhibitors. The structure and function of several archaeal RNAses that are involved in stable RNA processing and maturation have been reported. Examples include RNAse P (Pannucci et al., 1999), rRNA processing endonucleases (Russell et al., 1999), a tRNA splicing endonuclease (Lykke-Andersen & Garret, 1997; Li et al., 1998), and RNAse H (Muroya et al., 2001). The genes for some of these proteins and others containing RNAse-related domains are physically grouped and are distributed in this manner throughout the archaeal lineage (Koonin et al., 2001). This observation has been interpreted to mean that archaea have a eukaryotic-like exosome for RNA degradation.

In addition to enzymatic degradation, RNA also undergoes spontaneous hydrolysis at high temperature and moderately alkaline pH (Lindahl, 1967). This intrinsic instability may be of biological relevance to hyperthermophilic organisms, including both bacteria and archaea, where elevated growth temperatures create the potential for RNA hydrolysis. For example, in vitro transcription studies using a hyperthermophilic archaeal system demonstrated a role for additional factors mediating mRNA hydrolysis including magnesium and potassium (Hethke et al., 1999). In the work presented here, a method for the specific in vivo inhibition of RNA synthesis was developed for use on the hyperthermophile, *Sulfolobus solfataricus*, which is a member of the crenarchaeal subdivision of the archaea. A survey of mRNA stabilities of selected genes was then conducted, providing the first general estimate of mRNA turnover in this type of organism.

RESULTS

Identification of transcription inhibitors

Studies on the role of archaeal mRNA decay were enabled using a specific in vivo method for the elimination of transcription. Inhibitors of promoter-dependent archaeal transcription have not been reported; therefore, promoter of the *lacS* gene, *lacSp* (Haseltine et al., 1999a) was selected as a template for use in the identification of relevant compounds (Fig. 1A). *lacSp* consists of the consensus archaeal TATA box hexamer (CTTAAA) located 24 nt 5′ to the transcription start site of the *lacS* gene, where translation occurs at the coincident base (Haseltine et al., 1999a). Transcription of *lacSp* used a previously developed crude in vitro transcription system employing primer extension to assess transcript abundance and transcription start site specificity (Hudepohl et al., 1990). This system produced only limited levels of transcript from *lacSp* (Fig. 1B, lane 1); however, the combination of three changes significantly improved *lacSp* transcription (Fig. 1B, lanes 2–4). Changes included a reduction in the pH of

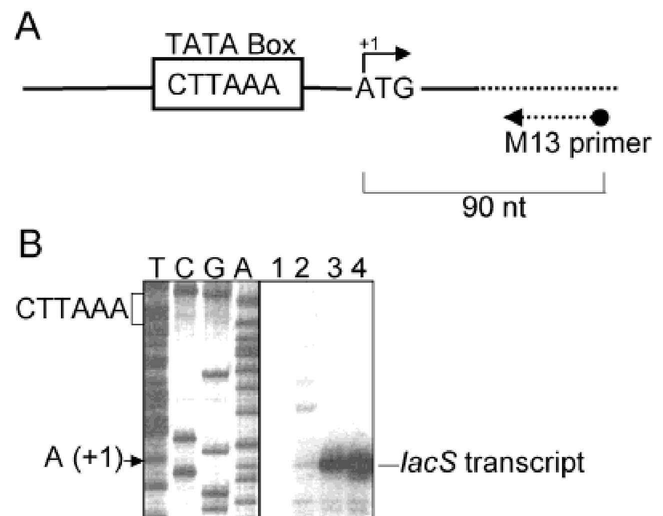

FIGURE 1. In vitro transcription of *lacSp*. **A:** Schematic of the *lacS* promoter (*lacSp*) template. The start point for transcription is located 23 bp 3′ to the end of the TATA box at the first base of the *lacS* start codon. Primer extension of in vitro transcription products results in formation of a 90-nt cDNA. **B:** Optimized in vitro transcription (IVT) of *lacSp*. Original IVT conditions, lanes 1 and 2; Optimized conditions, lanes 3 and 4. IVT conducted at 60 °C, lanes 1 and 3; or at 75 °C, lanes 2 and 4.

the transcription reaction (pH 8.0 to pH 6.0), an increase in the temperature of incubation (60 °C to 75 °C), and the use of whole-cell transcription extracts prepared from cells in balanced growth rather than in stationary phase. These modifications were employed in subsequent in vitro transcription assays for the identification of transcription inhibitors.

The modified in vitro transcription system was used to test the ability of eukaryotic and bacterial RNA synthesis inhibitors to block transcription of *lacSp* in vitro. Chromomycin, actinomycin D, distamycin, 1-10 phenanthroline, mithramycin, and daunorubicin completely inhibited transcription of *lacSp* (Fig. 2A). Novobiocin and the bacterial transcription inhibitor, rifampicin, had no effect on synthesis of *lacS* RNA in vitro. Of the compounds that were effective at blocking transcription in vitro, actinomycin D was the most potent, because it was effective at the lowest molarity. The in vivo application of any of the transcription inhibitors for the determination of mRNA half-lives necessitated that they survive the extreme growth conditions of *S. solfataricus*, notably high temperature (80 °C) and low pH (3.0). Consequently, the inhibitors were tested for thermal and acid stability. Those inhibitors shown to be effective at blocking *lacSp* transcription in vitro were subjected to pretreatment for 15 min and 180 min at 80 °C at a pH of 3.0. Pretreated samples were then tested by addition to *lacSp*-dependent in vitro transcription reactions. All the inhibitors tested retained inhibitory activity following the preincubation procedure (Fig. 2B).

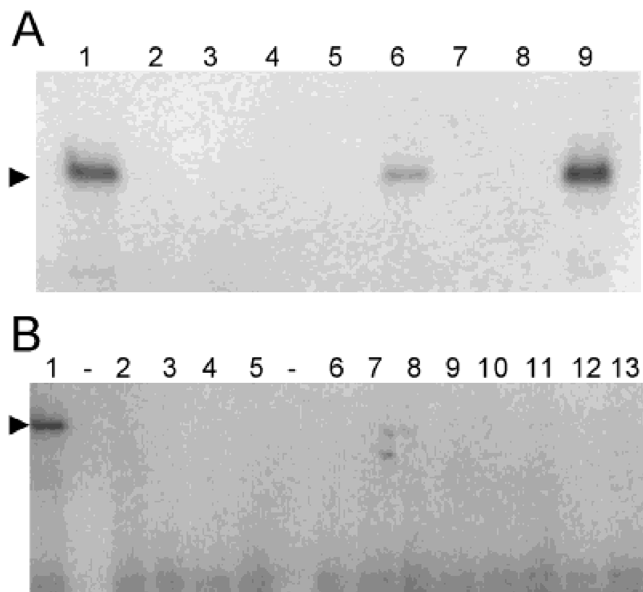

FIGURE 2. Transcription inhibitor screens. **A:** In vitro sensitivity of *lacSp* transcription. The lane number, inhibitor type, and amount were: 1: no inhibitor; 2: 200 μg/mL (0.17 mM) chromomycin; 3: 20 μg/mL (0.16 mM) actinomycin D; 4: 100 μg/mL (0.19 mM) distamycin; 5: 2 mg/mL (10 mM) 1-10 phenanthrolin; 6: 1 mg/mL (1.2 mM) rifampicin; 7: 200 μg/mL (0.18 mM) mithramycin; 8: 200 μg/mL (0.35 mM) daunorubicin; 9: 200 μg/mL (0.31 mM) novobiocin. **B:** Stability of transcription inhibitors. Inhibitors were preincubated at 80 °C and pH 3 for the times indicated and then tested in vitro. Samples in lanes 2, 4, 6, 8, 10, and 12 employed 15 min of pretreatment; samples in lanes 3, 5, 7, 9, 11, and 13 employed 180 min of pretreatment. Inhibitors were: no inhibitor (lane 1), daunorubicin (lanes 2 and 3), mithromycin (lanes 4 and 5), 1–10 phenanthroline (lanes 6 and 7), distamycin (lanes 8 and 9), actinomycin D (lanes 10 and 11), and chromomycin (lanes 12 and 13).

Inhibition of RNA synthesis in vivo

Incorporation of radiolabeled uracil or uridine into TCA-precipitable material is a general method used to determine the in vivo rate of total RNA synthesis (Pato et al., 1973). Preliminary experiments using tritiated uridine showed that *S. solfataricus* does not incorporate uridine to significant levels during growth in a sucrose minimal medium. In contrast, radiolabeled uracil was incorporated efficiently at a rate of 1.5×10^3 cpm/min, which is equivalent to 0.1 pmol/min/2.7×10^6 cells. To measure the effectiveness of actinomycin D exposure on radiolabeled uracil incorporation, the inhibitor was tested at several concentrations (Fig. 3). The highest concentration (10 μg/mL) completely blocked uracil incorporation within 10 min after addition. The lower concentration of actinomycin D (5 μg/mL) reduced the rate of uracil incorporation to 50% of that of untreated cells. Parallel observations on cell growth and appearance were performed to assess the physiological consequences of inhibitor exposure (Fig. 3, inset). No changes in growth rate were observed for 2 h after addition of actinomycin D (10 μg/mL); however, growth ceased following this period. No changes in cell morphology were observed within the initial 2-h treatment period. At times thereafter, morphological changes became apparent, including cell swelling and loss of phase contrast. As such changes resemble those

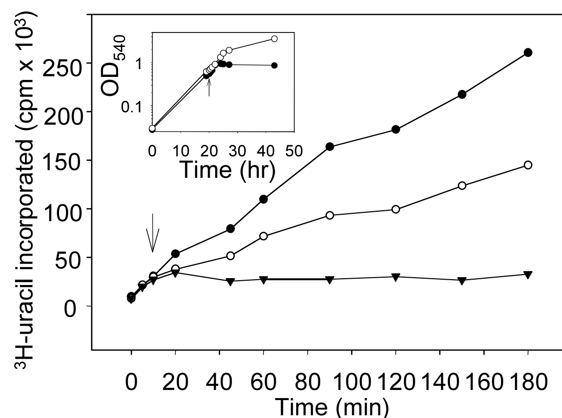

FIGURE 3. Effect of actinomycin D on RNA synthesis and growth. Measurement of ^{3}H-uracil incorporation into bulk RNA following culture treatment with actinomycin D at 5.0 μg/mL (open circles), 10.0 μg/mL (closed inverted diamonds), or untreated (closed circles). Actinomycin D was added at the time indicated by the arrow. Inset: Growth response of treated cells. Untreated culture (open circles), culture treated with 10 μg/mL of actinomycin D (closed circles).

of cells experiencing unbalanced growth, mRNA decay measurements were confined to the initial 2-h treatment period.

Half-lives of archaeal mRNAs

To obtain a general assessment of the half-lives of mRNAs from this organism, a survey of mRNA decay rates was conducted. Transcripts were selected that encoded proteins representing a wide range of cellular functions, including transcription, polysaccharide hydrolysis, amino acid biosynthesis, oxidative stress, and glycolysis. These included both paralogs of the TFIIB eukaryal basal transcription factor, TFB1 and TFB2 (*tfb1*, Qureshi & Jackson, 1998; and *tfb2*); alpha-glucosidase (*malA*; Rolfsmeier et al., 1998), which catalyzes hydrolysis of maltose and maltodextrins; one of three glutamine synthetase paralogs (*gln1*); superoxide dismutase (*sod*; Ursby et al., 1999); and one of three paralogs of glucose dehydrogenase (*dhg1*), which catalyzes the first committed step in the Entner-Doudoroff pathway. To determine mRNA half-life, mRNA levels of the selected genes were determined by northern analysis following actinomycin D treatment. Transcript levels were normalized to amounts of a structural RNA called 7S RNA as described (Cheung et al., 1997). Northern blots were probed simultaneously with antisense RNA riboprobes complementary to the mRNA of interest and to 7S RNA. Representative autoradiograms are shown for each mRNA representing one of three experiments used to derive transcript half-lives (Fig. 4). The half-lives of the RNA transcripts were measured over a maximum time range of 120 min. Band intensities for each mRNA for each sample time were determined by densitometry and normalized to those for 7S RNA in the same lane. The percent of the initial levels of each transcript prior to actinomycin D treatment was plotted for each of the sample times that were analyzed (Fig. 5). The slopes of the lines for three separate experiments were used to derive mean values and standard deviations for the half-lives of each mRNA. Transcript half-lives were at least 2 h for both *tfb1* and *sod*. The half-lives of the other transcripts were: *dhg1*, 37.5 min ($SD = 7.5$); *malA*, 25 min ($SD = 10.7$); *tfb2*, 13.5 min ($SD = 4.5$); and *gln1*, 6.3 min ($SD = 1.2$) and a minimum average half-life of 54 min.

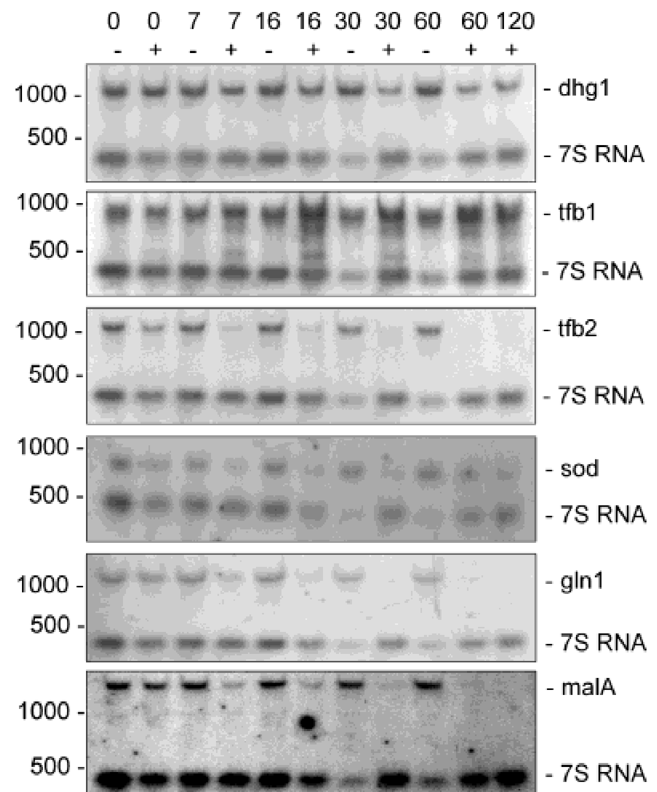

FIGURE 4. Decay of *S. solfataricus* mRNAs. Autoradiograms of northern blots were probed simultaneously with riboprobes specific for each target mRNA and the 7S RNA. RNA samples obtained at the indicated times were from untreated cultures (−) or actinomycin D-treated cultures (+).

DISCUSSION

Rapid mRNA removal enables use of transcription initiation as a critical point of control for gene expression. Like bacteria, archaea such as *S. solfataricus* are prokaryotes; however, mRNA chemical half-lives in this organism ranged from minutes to hours, longer than expected for a typical bacterium. Normalization of the average mRNA chemical half-life in *S. solfataricus* ($T_{1/2}$, 54 min) to its cellular generation time (g, 360 min) results in a ratio of 0.15. Comparison of this $T_{1/2}/g$ ratio to that of other organisms including *E. coli*, 0.03 ($T_{1/2}$, 1 min; g, 30 min; Coburn & Mackie, 1999), *S. cerevisiae*, 0.25 ($T_{1/2}$, 23 min; g, 90 min; Wang et al., 2002),

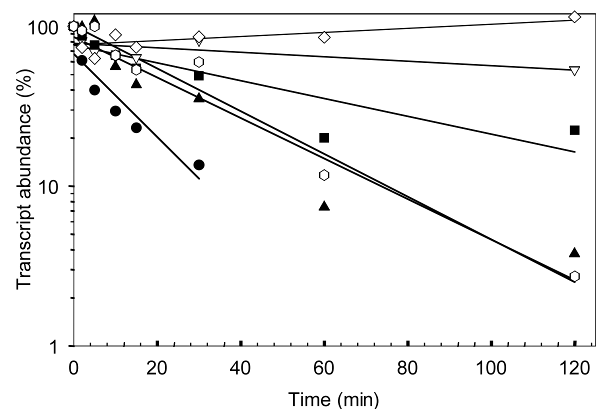

FIGURE 5. Rates of mRNA decay. Values for remaining levels of each mRNA are presented as a percentages of untreated samples prior to actinomycin D addition. Symbols are: *tfb1*, open diamond; *sod*, inverted open triangle; *dhg1*, closed square; *malA*, open circle; *tfb2*, closed triangle; and *gln1*, closed circle.

and cultured mammalian cells, 1.33 ($T_{1/2}$, 16 h; *g*, 12 h; Singer & Penman, 1973) further distinguishes between model prokaryotic organisms, and places *S. solfataricus* close to *S. cerevisiae*. The occurrence of stable *S. solfataricus* mRNAs also is surprising in light of the elevated growth temperature of the organism. Studies on the transcription apparatus of another hyperthermophile, *Pyrococcus furiosus*, showed that high temperatures accelerate the chemical hydrolysis of mRNA (Hethke et al., 1999). Consequently, the findings presented here indicate that the intracellular composition of *S. solfataricus* must constrain the natural hydrolytic instability of RNA (Lindahl, 1967). This constraint could reflect a particular ionic composition that passively inhibits RNA cleavage or, by analogy to eukaryotes, it could indicate the existence of positively acting factors that block RNA turnover.

Increased mRNA stability in *S. solfataricus* elevates the significance of protein synthesis as a controlling element for the expression of its genes. Recent studies on aminoacyl tRNA synthetases have revealed that methanogenic archaea employ unique strategies for maintaining aminoacylated tRNA pools (Stathopoulos et al., 2001). An additional consequence of greater mRNA stability on gene expression in *S. solfataricus* is the increased importance of dilution of undegraded mRNA by cell division as a means of readjusting transcript abundance. Because the minimum generation time is 4–6 h, the period of readjustment necessary to reduce the concentration of the more stable mRNAs would be prolonged. Mechanisms to regulate translation would then become necessary to accomplish reduced gene product abundance. If translational control exists, however, it must accommodate the frequent lack of untranslated leader RNAs that results in a coincidence of start sites for transcription and translation (Tolstrup et al., 2000). Prolonged mRNA stability may also be of importance in the regulation of the *Sulfolobus* cell cycle (Hjort & Bernander, 2001).

A range of mRNAs was tested in this study, including members from several areas of cell biology. These included representatives of several paralogous groups of genes. Surprisingly, the stabilities of the mRNAs of the two transcription factor TFIIB paralogs were quite dissimilar. The half-life of the transcript for *tfb1* was in excess of 2 h whereas that of *tfb2* was 18 min. TFB associates with the TATA binding protein, TBP, at the archaeal promoter (TATA box) and subsequently recruits RNA polymerase to the DNA (Bell et al., 1999; Bartlett et al., 2000). Because *tfb2* mRNA is relatively unstable, conditions that perturb *tfb2* mRNA synthesis could lead to differences in its abundance. If the levels of TFB2 parallel this pattern, conditions could exist where the relative abundance of the two TFBs vary, resulting in differences in the expression of genes dependent on them for transcription. This possibility is under investigation.

S. solfataricus encodes three paralogs of glutamine synthetase termed *gln1*, *gln2*, and *gln3*, which are encoded by ORFs SSO336, SSO2440, and SSO2554, respectively (She et al., 2001). These genes encode proteins with 26 to 34% amino acid sequence identity to each other and have similar lengths, mass, and isoelectric points. Northern blot analysis using riboprobes derived from each of the coding sequences indicated that only one, *gln1*, encoded an mRNA produced in sufficient quantity to allow its detection under the growth conditions employed. Conditions of nitrogen sufficiency and deficiency wait to be determined for this organism; therefore, it remains possible that the other paralogs do produce detectable transcripts but are synthesized under conditions distinct from those of *gln1*. The third set of paralogous genes were represented by *dhg1*, encoding one of three paralogs of glucose dehydrogenase. This enzyme catalyzes the first committed step in a modified Entner Doudoroff pathway for glucose catabolism, producing gluconic acid in an NADH-dependent reaction (Danson & Hough, 1992). Other genes in this survey included the iron-dependent superoxide dismutase, *sod* (Ursby et al., 1999), which produces an activity involved in maintaining proper levels of oxygen free radicals. The cytoplasmic alpha-glucosidase (*malA*) catalyzes hydrolysis of exogenous glucose polymers (Rolfsmeier & Blum, 1995; Rolfsmeier et al., 1998), and was used to represent secondary metabolism.

The archaeal RNAP can transcribe DNA at alkaline pH and high temperature without undergoing specific initiation at promoter sequences (Zillig et al., 1979). This activity is sensitive to heparin and actinomycin D and resistant to specific inhibitors of the bacterial RNAP such as rifampicin and streptolydigin (Sippel & Hartmann, 1968). The studies reported here extend these earlier data to show that *lacS* promoter-dependent transcription exhibits similar responses to actinomycin D and rifampicin. In addition, other compounds that block eukaryal transcription, including chromomycin and distamycin (Orfeo et al., 1999), 1-10 phenanthroline (Logan et al., 1989), mithramycin (Mazumder et al., 1994), and daunorubicin (Kriebardis et al., 1987), were tested for utility in the optimized *S. solfataricus* in vitro transcription system. All these compounds form a complex with DNA and either prevent the binding of RNAP or promote its displacement from the promoter, and all were found to be effective in vitro inhibitors of transcription of the *lacSp* using the *S. solfataricus* system. Novobiocin, in contrast, which has been shown to block initiation of transcription directed by eukaryal RNAP I, II, and III on linear templates (Webb et al., 1987), had no effect on transcription of *lacSp* in vitro. The availability of a specific method for measuring mRNA half-life in *S. solfataricus* will enable future studies directed towards elucidating the mechanism of mRNA degradation in this member of the archaea. The finding of prolonged

chemical half-lives for several *S. solfataricus* mRNAs suggests this organism may employ alternative mechanisms for the regulation of gene expression and mRNA degradation.

MATERIALS AND METHODS

Archaeal strains and cultivation

S. solfataricus, strain 98/2, was grown at 80 °C in batch culture as described previously (Rolfsmeier & Blum, 1995). The basal salts medium of Allen (1959) as modified by Brock et al. (1972), supplemented with sucrose 0.2% (w/v) as the sole carbon and energy source, was used for studies on mRNA levels and stability. Tryptone (0.2%) supplementation of this medium was used for preparation of cell extracts for in vitro transcription. Growth was monitored at a wavelength of 540 nm using a Cary 50 Bio, UV-visible spectrophotometer (Varian). Cell-free extracts and RNA samples were prepared from 0.5-L cultures harvested at the indicated times.

Uracil incorporation

Cultures were grown to early exponential phase equivalent to a cell density of 6.8×10^7 cells/mL ($OD_{540} = 0.17$) and 0.25-mL volumes were transferred to capped polypropylene microcentrifuge tubes and equilibrated for 5 min at 75 °C. Radiolabeled uracil [5,6-^{3}H, 33.1 Ci/mmol; NEN) was added to a final concentration of 105 μCi/mL. An optimal ratio of radiolabeled uracil to cells sufficient to ensure the time-linear synthesis of RNA over a 3-h period was found to be 189 μCi/10^8 cells. Tubes were inverted at 10-min intervals to aerate the cells. Samples (10 μL) were collected in duplicate at the indicated times and transferred to tubes containing 0.5-mL volumes of unlabeled cells (4×10^8 cells/mL), added to promote centrifugal recovery of TCA precipitates. Cold 50% (w/v) TCA was added to each sample to a final TCA concentration of 11.5% (w/v). After 5 min on ice, the samples were centrifuged at $15{,}000 \times g$, and the supernatant was discarded. The pellet was resuspended in 25 μL of TE and radioactivity was measured in an LS 1701 Liquid Scintillation System (Beckman) using an open window. Radiolabeled uracil was added 10 min after addition of actinomycin D.

Molecular biology methods

Recombinant molecular biology procedures for DNA cloning, PCR, and plasmid transformation were performed as described (Rockabrand et al., 1998; Haseltine et al., 1999a). DNA sequencing was as described (Rolfsmeier et al., 1998). DNA and RNA concentrations were measured using either a DyNA Quant 200 fluorometer (Hoefer) or a UV-visible spectrophotometer Genesys 2 (Spectronics). All manipulations of RNA were as described (Haseltine et al., 1999b). Protein concentrations were measured using the BCA Protein Assay Reagent Kit (Pierce). Unless otherwise indicated, all chemicals were obtained from common chemical suppliers. Plasmid DNA inserts were sequenced using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham) following the instructions of the manufacturer. The primer 5′-CCCTGGTG TTCCCATTTCTGATTGAAATCC was used to generate the sequencing ladder for mapping the start site of transcription of *lacSp*. cDNA samples originating from the in vitro transcription reactions and DNA sequencing reaction products were separated on preequilibrated 8% (w/v) denaturing polyacrylamide sequencing gels as described (Rolfsmeier et al., 1998).

In vitro transcription

Cell-free extracts were prepared as described (Hudepohl et al., 1990) with modifications. Improved *lacSp* transcription was obtained using extracts from cells harvested in mid-exponential phase followed by resuspension in an alternative in vitro transcription buffer (IVTB; 50 mM MES, pH 6, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT). Typical extracts had protein concentrations of 10–20 mg/mL and were processed and stored as described (Hudepohl et al., 1990). These extracts retained potency for up to 4 months. In vitro transcription templates consisted of linearized *S. solfataricus* promoters cloned in pUC19. The *lacSp* template consisted of a 220 pb PCR product obtained with primers 5′-GGATCCCAATACCTAGGAG GAGTAGC and 5′-GGCCTGGGAGCATGCAAACCTAAAGC inserted at the *Bam*HI and *Sph*I sites. The *lacSp* in vitro promoter template was linearized by digestion with *Afl*III.

In vitro transcription reaction mixtures contained 0.2 mM rATP, 0.1 mM each of rCTP, rGTP, and rUTP, a volume of cell extract corresponding to 100–200 μg of total protein, 0.4 μg linearized promoter template in a final volume of 50 μL adjusted with DEPC-treated water and either IVTB or the buffer employed previously (Hudepohl et al., 1990). Reactions were initiated by cell extract addition, incubated for 10 min, chilled on ice, adjusted to 25 mM EDTA, pH 8, 0.5 mg/mL yeast tRNA, and extracted three times with equal volumes of phenol:chloroform. Transcription products were recovered by ethanol precipitation and template DNA was removed by treatment with 20 U of DNAse I (Pharmacia) at room temperature for 0.5 h followed by adjustment to 25 mM EDTA and extraction with equal volumes of phenol:chloroform and chloroform:isoamyl alcohol. The RNA was recovered by ethanol precipitation, lyophilized, resuspended in DEPC-treated water, and stored at −80 °C.

Primer extension analysis

In vitro transcription products were primer extended using the primer 5′-AGCGGATAACAATTTCACACAGGAAACAGC, which is complementary to the vector and provides a common 5′ cDNA end. Primer extension oligonucleotides were labeled at the 5′ end with [γ-^{32}P]-ATP using T4 polynucleotide kinase (NEB) as described (Triezenberg, 1992). The labeling reaction was terminated by EDTA addition followed by heating at 65 °C. The labeled primer was purified using a Sep-pak cartridge (Waters), dried, and resuspended in 10 μL 10 mM Tris-Cl, pH 8.0, 1 mM EDTA. A typical reaction yielded 10 μL of 10^6 cpm/μL labeled oligo, and 1 μL of this oligo was used for each reverse transcription reaction. Reverse transcription was as described (Triezenberg, 1992) with modifications. In vitro transcribed RNA was resuspended in 150 mM potassium chloride, 10 mM Tris-Cl, pH 8.3, and 1 mM EDTA, combined with 10^6 cpm of labeled primer extension primer, heated at 65 °C for 90 min and cooled to allow primer an-

nealing. The mixture was adjusted to 20 mM Tris-Cl, pH 8.3, 10 mM $MgCl_2$, 0.5 mM DTT, 0.15 mg/mL actinomycin D, 0.15 mM dNTPs, and 0.33 μL of AMV reverse transcriptase (Pharmacia) was added. The reaction was incubated for 1 h at 42 °C and terminated by adjustment to 17.5 ng/mL salmon sperm DNA, 14 ng/mL RNase A with incubation for 15 min at 37 °C. The reaction was extracted with phenol:chloroform and primer extended DNA was recovered by ethanol precipitation, dried, and resuspended in the Stop Solution of the T7 Sequenase version 2.0 DNA sequencing kit (Amersham).

Screening of transcription inhibitors

Stock solutions of the inhibitors were: chromomycin, 20 mg/mL in ethanol; actinomycin D, 1 mg/mL in 50% ethanol; distamycin, 20 mg/mL in ethanol; 1-10 phenanthrolin, 100 mg/mL in ethanol; rifampicin, 50 mg/mL in chloroform; mithramycin, 10 mg/mL in methanol; daunorubicin, 10 mg/mL in water; and novobiocin, 10 mg/mL in water. Inhibitors were added to the in vitro transcription reactions in the amounts indicated; addition of solvents alone had no effect on the in vitro reaction. Preincubation of transcription inhibitors to assess thermal and acid stability was conducted at the following inhibitor concentrations: chromomycin, 5 mg/mL; actinomycin D, 0.5 mg/mL; distamycin, 2.5 mg/mL; 1-10 phenanthroline, 50 mg/mL; mithramycin, 5 mg/mL; and daunorubicin, 5 mg/mL.

Northern blot analysis

Northern hybridization analysis using antisense riboprobes was performed as described (Haseltine et al., 1999a). RNAs were detected by autoradiography on X-OMAT AR film (Kodak). Digital images were acquired using a Gel Documentation System GDS7600 (UVP). Scanning densitometry of the images was performed using GelBase-Pro software (UVP). Most riboprobes were prepared using PCR-amplified segments of the selected genes from genomic DNA of *S. solfataricus* strain 98/2 identified by ORF number (She et al., 2001) and cloned into plasmid pT7T3/18U (Pharmacia). The *gln1* construct was obtained from SSO0336 with primers 5′-TTGTAGATACTGCAGACAAGGTCCAAACTTTG and 5′-CTCATCTATCTAAAGCTTCATTTAATGATCTAGG, and the 602-nt product was cloned as a *Pst*I-*Hin*dIII fragment. The *sod* construct was obtained from SSO0316, with primers 5′-ATTACCCTACAATCTAGATGCATTAGAACC and 5′-TAC ATTCCACCAAGCTTTAACGTAATCAGC, and the resulting 520-bp product was cloned as an *Xba*I-*Hin*dIII fragment. The 7S RNA was amplified from SSO0316 with the primers 5′-CCTTTATCAATACTCTTAAAAATAATAAGG and 5′-TCAC TCTGGCTGTTGACCAGCGAACGCTGG, and the 306-bp product was blunt cloned at the *Hin*cII site. The *dhg1* construct was comprised of a 460-bp *Bam*HI-*Eco*RI internal fragment subcloned as such from the full-length cloned copy of the gene. The *malA* probe was as described (Rolfsmeier et al., 1998). The *tfb1* construct was amplified from SSO0446 using primers 5′-TTTGTCTAGAGAAAATAAATCCGTATCC and 5′-TAATAGTCTAAAGCTTCTCGCTACTTCC, and the resulting 590-bp product cloned as a *Xba*I and *Hin*dIII fragment. The *tfb2* probe was obtained from SSO0946 with primers 5′-AGCTCAAATCTAGAATTGCCAGAACATGTC and 5′-AG AAGCTTCGGTAATATCTAATGAATCTGC, and the 480-bp product cloned as a *Xba*I-*Hin*dIII site. The composition of all inserts were verified by DNA sequencing. For use in riboprobe synthesis, plasmids were linearized by digestion with either *Hin*dIII (*7S rRNA*), *Eco*RI (*sod*), *Sma*I (*gln1*, *tfb1*, *tfb2*), *Xba*I (*dhg1)*, or *Sac*I (*malA*). T3 RNA polymerase (*sod*, *gln1*, *tfb1*, *tfb2*, *malA*) or T7 RNA polymerase (*7S RNA*, *dhg1*) were used for transcription from the appropriate promoter following the manufacturer's instructions to produce a ^{32}P-labeled antisense RNA.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation Grants MCB-9974453 and MCB-0085216.

Received April 2, 2002; returned for revision June 14, 2002; revised manuscript received July 2, 2002

REFERENCES

Allen MB. 1959. Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch Mikrobiol 32*:270–277.
Arnold TE, Yu J, Belasco JG. 1998. mRNA stabilization by the ompA 5′ untranslated region: Two protective elements hinder distinct pathways for mRNA degradation. *RNA 4*:319–330.
Bartlett MS, Thomm M, Geiduschek EP. 2000. The orientation of DNA in an archaeal transcription initiation complex. *Nat Struct Biol 7*:782–785.
Bell SD, Kosa PL, Sigler PB, Jackson SP. 1999. Orientation of the transcription preinitiation complex in archaea. *Proc Natl Acad Sci USA 96*:13662–13667.
Blum E, Py B, Carpousis JAJ, Higgins CF. 1997. Polyphosphate kinase is a component of the *Escherichia coli* RNA degradosome. *Mol Microbiol 26*:387–398.
Brock TD, Brock KM, Belly RT, Weiss RL. 1972. *Sulfolobus*: A genus of sulfur oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol 84*:54–68.
Brown JW, Reeve JN. 1985. Polyadenylated non-capped RNA from the archaebacterium *Methanococcus vannielii*. *J Bacteriol 162*: 909–917.
Brown JW, Reeve JN. 1986. Polyadenylated RNA isolated from the archaebacterium *Halobacterium halobium*. *J Bacteriol 166*: 686–688.
Cheung J, Danna KJ, O'Connor EM, Price LB, Shand RF. 1997. Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. *J Bacteriol 179*:548–551.
Coburn GA, Mackie GA. 1999. Degradation of mRNA in *Escherichia coli*: An old problem with some new twists. *Prog Nucleic Acid Res 62*:55–108.
Danson MJ, Hough DW. 1992. The enzymology of archaebacterial pathways of central metabolism. *Biochem Soc Symp 58*:7–21.
Grunberg-Manago M. 1999. Messenger RNA stability and its role in control of gene expression in bacteria and phages. *Ann Rev Genet 33*:193–227.
Hambraeus G, Persson M, Rutberg B. 2000. The *aprE* leader is a determinant of extreme mRNA stability in *Bacillus subtilis*. *Microbiol 146*:3051–3059.
Haseltine C, Montalvo-Rodriguez R, Bini E, Carl A, Blum P. 1999a. Coordinate transcriptional control in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol 181*:3920–3927.
Haseltine C, Montalvo-Rodriguez R, Carl A, Bini E, Blum P. 1999b. Extragenic pleiotropic mutations that repress glycosyl hydrolase expression in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *Genetics 152*:1353–1361.
Hennigan AN, Reeve JN. 1994. mRNAs in the methanogenic archaeon *Methanococcus vannielii*: Numbers, half-lives and processing. *Mol Microbiol 11*:655–670.

Hethke C, Bergerat A, Hausner W, Forterre P, Thomm M. 1999. Cell-free transcription at 95 degrees: Thermostability of transcriptional components and DNA topology requirements of *Pyrococcus* transcription. *Genetics 152*:1325–1333.
Hjort K, Bernander R. 2001. Cell cycle regulation in the hyperthermophilic crenarchaeon *Sulfolobus acidocaldarius*. *Mol Microbiol 40*:225–234.
Hudepohl U, Reiter WD, Zillig W. 1990. *In vitro* transcription of two rRNA genes of the archaebacterium *Sulfolobus* sp. B12 indicates a factor requirement for specific initiation. *Proc Natl Acad Sci USA 87*:5851–5855.
Koonin EV, Wolf YI, Aravind L. 2001. Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. *Genome Res 11*:240–252.
Kriebardis T, Meng D, Aktipis S. 1987. Inhibition of the RNA polymerase-catalyzed synthesis of RNA by daunomycin. Effect of the inhibitor on the late steps of RNA chain initiation. *J Biol Chem 262*:12632–12640.
Li H, Trotta CR, Abelson J. 1998. Crystal structure and evolution of a transfer RNA splicing enzyme. *Science 280*:279–284.
Lindahl T. 1967. Irreversible heat inactivation of transfer ribonucleic acids. *J Biol Chem 242*:1970–1973.
Logan K, Zhang J, Davis EA, Ackerman S. 1989. Drug inhibitors of RNA polymerase II transcription. *DNA 8*:595–604.
Lykke-Andersen J, Garrett RA. 1997. RNA–protein interactions of an archaeal homotetrameric splicing endoribonuclease with an exceptional evolutionary history. *EMBO J 16*:6290–6300.
Mazumder A, Perrin DM, McMillin D, Sigman DS. 1994. Interactions of transcription inhibitors with the *Escherichia coli* RNA polymerase-lacUV5 promoter open complex. *Biochem 33*:2262–2268.
Mitchell P, Tollervey D. 2000. mRNA stability in eukaryotes. *Curr Opin Genet Dev 10*:193–198.
Muroya A, Tsuchiya D, Ishikawa M, Haruki M, Morikawa M, Kanaya S, Morikawa K. 2001. Catalytic center of an archaeal type 2 ribonuclease H as revealed by X-ray crystallographic and mutational analyses. *Protein Sci 10*:707–714.
Nilsson G, Belasco JG, Cohen SN, von Gabain A. 1984. Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. *Nature 312*:75–77.
O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E, Kushner SR. 1995. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci USA 92*:1807–1811.
Orfeo T, Chen L, Huang W, Ward G, Bateman E. 1999. Distamycin A selectively inhibits Acanthamoeba RNA synthesis and differentiation. *Biochem Biophys Acta 1446*:273–285.
Pannucci JA, Haas ES, Hall TA, Harris JK, Brown JW. 1999. RNase P RNAs from some Archaea are catalytically active. *Proc Natl Acad Sci USA 96*:7803–7808.
Pato ML, Bennett PM, von Meyenburg K. 1973. Messenger ribonucleic acid synthesis and degradation in *Escherichia coli* during inhibition of translation. *J Bacteriol 116*:710–718.
Qureshi SA, Jackson SP. 1998. Sequence-specific DNA binding by the *S. shibatae* TFIIB homolog, TFB, and its effect on promoter strength. *Mol Cell 1*:389–400.
Rockabrand D, Livers K, Austin T, Kaiser R, Jensen D, Burgess R, Blum P. 1998. Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli*. *J Bacteriol 180*:846–854.
Rolfsmeier M, Blum P. 1995. Purification and characterization of a maltase from the extremely thermophilic Crenarchaeote *Sulfolobus solfataricus. J Bacteriol 177*:482–485.
Rolfsmeier M, Haseltine C, Bini E, Clark A, Blum P. 1998. Molecular characterization of the alpha-glucosidase gene (*malA*) from the hyperthermophilic archaeon *Sulfolobus solfataricus*. *J Bacteriol 180*:1287–1295.
Ross J. 1995. mRNA stability in mammalian cells. *Microbiol Rev 59*:423–450.
Russell AG, Ebhardt H, Dennis PP. 1999. Substrate requirements for a novel archaeal endonuclease that cleaves within the 5′ external transcribed spacer of *Sulfolobus acidocaldarius* precursor rRNA. *Genetics 152*:1373–1385.
Sarkar N. 1997. Polyadenylation of mRNA in prokaryotes. *Annu Rev Biochem 66*:173–197.
Seiser C, Posch M, Thompson N, Kuhn LC. 1995. Effect of transcription inhibitors on the iron-dependent degradation of transferrin receptor mRNA. *J Biol Chem 270*:29400–29406.
She Q, Singh RK, Confalonieri F, Zivanovic Y, Allard G, Awayez MJ, Chan-Weiher CC, Clausen IG, Curtis BA, De Moors A, Erauso G, Fletcher C, Gordon PM, Heikamp-de Jong I, Jeffries AC, Kozera CJ, Medina N, Peng X, Thi-Ngoc HP, Redder P, Schenk ME, Theriault C, Tolstrup N, Charlebois RL, Doolittle WF, Duguet M, Gaasterland T, Garrett RA, Ragan MA, Sensen CW, Van der Oost J. 2001. The complete genome of the Crenarchaeote *Sulfolobus solfataricus* P2. *Proc Natl Acad Sci USA 98*:7835–7840.
Shyu AB, Belasco JG, Greenberg ME. 1991. Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. *Genes Dev 5*:221–231.
Singer RH, Penman S. 1973. Messenger RNA in HeLa cells: Kinetics of formation and decay. *J Mol Biol 78*:321–324.
Sippel A, Hartmann G. 1968. Mode of action of rafamycin on the RNA polymerase reaction. *Biochim Biophys Acta 157*:218–219.
Stathopoulos C, Kim W, Li T, Anderson I, Deutsch B, Palioura S, Whitman W, Soll D. 2001. *Methanococcus jannaschii* prolyl-cysteinyl-tRNA synthetase possesses overlapping amino acid binding sites. *Proc Natl Acad Sci USA 98*:14292–14297.
Steege D. 2000. Emerging features of mNRA decay in bacteria. *RNA 6*:1079–1090.
Tharun S, Parker R. 1997. Mechanisms of mRNA turnover in eukaryotics cells. In Harford JB, Morris DR, eds. *mRNA metabolism and post-transcriptional gene regulation*. New York: Wiley-Liss. pp 181–199.
Tolstrup N, Sensen CW, Garrett RA, Clausen IG. 2000. Two different and highly organized mechanisms of translation initiation in the archaeon *Sulfolobus solfataricus*. *Extremophiles 4*:175–179.
Triezenberg SJ. 1992. Preparation and analysis of RNA. In: Ausubel FM, Brent R, Kingston RE, More DD, Seidman JG, Smith JA, Struhl K. eds. *Current protocols in molecular biology*. New York: John Wiley. pp 4.8.1–4.8.5.
Ursby T, Adinolfi BS, Al-Karadaghi S, De Vendittis E, Bocchini V. 1999. Iron superoxide dismutase from the archaeon *Sulfolobus solfataricus*: Analysis of structure and thermostability. *J Mol Biol 286*:189–205.
Vanzo NF, Li YS, Py P, Blum E, Higgins CF, Raynal LC, Krisch HM, Carpousis AJ. 1998. Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome. *Genes Dev 12*:2770–2781.
Volkl P, Markiewicz P, Baikalov C, Fitz-Gibbon S, Stetter KO, Miller JH. 1996. Genomic and cDNA sequence tags of the hyperthermophilic archaeon *Pyrobaculum aerophilum*. *Nucleic Acids Res 24*:4373–4378.
Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO. 2002. Precision and functional specificity in mRNA decay. *Proc Natl Acad Sci USA 99*:5860–5865.
Warner JR, Gorenstein C. 1978. Yeast has a true stringent response. *Nature 275*:338–339.
Webb ML, Maguire KA, Jacob ST. 1987. Novobiocin inhibits initiation of RNA polymerase II-directed transcription of the mouse metallothionein-I gene independent of its effect on DNA topoisomerase II. *Nucleic Acids Res 15*:8547–8560.
Woese CR, Fox GE. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proc Natl Acad Sci USA 74*: 5088–5090.
Xu F, Lin-Chao S, Cohen SN. 1993. The *Escherichia coli pcnB* gene promotes adenylation of antisense RNAI of ColE1-type plasmids in vivo and degradation of RNAI decay intermediates. *Proc Natl Acad Sci USA 90*:6756–6760.
Xu N, Chen CY, Shyu AB. 1997. Modulation of the fate of cytoplasmic mRNA by AU-rich elements: Key sequence features controlling mRNA deadenylation decay. *Mol Cell Biol 17*:4611–4621.
Zillig W, Stetter KO, Janekovic D. 1979. DNA-dependent RNA polymerase from the archaebacterium *Sulfolobus acidocaldarius*. *Eur J Biochem 96*:597–604.