

# Protein biogenesis in Archaea: addressing translation initiation using an *in vitro* protein synthesis system for *Haloferax volcanii*

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## Introduction

The mosaic character of archaeal protein translation is immediately apparent when one considers what is known of translation initiation in Archaea (Condo *et al.*, 1999; Tolstrup *et al.*, 2000; Benelli *et al.*, 2003; Londei, 2005). On the one hand, archaeal mRNAs often encode Shine–Dalgarno (SD) motifs within their 5'-untranslated regions (5'-UTR). In Bacteria, the SD motif is thought to position the ribosome on an mRNA just upstream of the start codon through an interaction with a complementary sequence in the small ribosomal subunit RNA (Shine & Dalgarno, 1974). On the other hand, archaeal mRNA may also be leaderless, i.e. where the leader sequence is either very short or even completely absent. The mechanism by which translation of leaderless transcripts is initiated remains poorly understood, although it may share common properties with the 'ribosome-scanning' approach used in Eukarya (Kozak, 1989; Londei, 2005). The similar initiation factor profiles of Archaea and Eukarya also point to parallels in the initiation

## Abstract

Translation initiation in Archaea combines aspects of the parallel process in Eukarya and Bacteria alongside traits unique to this domain. To better understand translation initiation in Archaea, an *in vitro* translation system from the haloarchaeon *Haloferax volcanii* has been developed. The ability to translate individual mRNAs both under the conditions used in previously developed poly(U)-dependent poly(Phe) synthesis systems as well as under physiological conditions was shown. Using the *H. volcanii* system, mRNAs preceded by either 'strong' or 'weak' Shine–Dalgarno (SD) motifs, or completely lacking leader sequences were effectively translated. The *in vitro* haloarchaeal system also successfully translated mRNA from Bacteria, again either presenting a SD initiation motif or completely lacking a leader sequence. Thus, the ability to translate individual mRNAs *in vitro* offers a system to address translation initiation as well as other aspects of protein biogenesis in Archaea.

of translation in both domains (Dennis, 1997; Bell & Jackson, 1998; Londei, 2005).

The first experimental proof that both SD motif-bearing and leaderless mRNAs are indeed recognized in Archaea relied on an *in vitro* protein translation system reconstituted from the thermoacidophile *Sulfolobus solfataricus* capable of efficient and accurate translation of mRNA encoding full-length polypeptides of well over 100 amino acid residues (Condo *et al.*, 1999). In using this system to address the roles of SD motifs in a bicistronic mRNA construct, it was shown that the motif preceding each mRNA is required for translation of the corresponding downstream sequence. Disruption of either SD motif prevented translation of the corresponding message unless the entire leader sequence was removed, in which case translation was once again possible. Later *in vivo* studies confirmed that haloarchaea can also initiate translation through ribosome binding to SD motifs, although the effects of SD motif disruption were less drastic in the halophilic cell than in the *in vitro* thermoacidophilic system (Sartorius-Neef & Pfeifer, 2004).

Towards a better understanding of archaeal translation initiation, the availability of an *in vitro* protein translation system derived from haloarchaeal components would be of extreme value. Unfortunately, despite the fact that amongst the most advanced tools for studying Archaea at the molecular level currently in use are designed for work with halophilic species (Soppa, 2006), the reconstitution of protein translation using haloarchaeal components has met with only limited success. While efficient *in vitro* poly(U)-dependent poly(Phe) synthesis systems using haloarchaeal ribosomes have been long available (Bayley & Griffiths, 1968; Saruyama & Nierhaus, 1985; Sanz *et al.*, 1988), the limited number of later attempts at creating a system capable of true *in vitro* halophilic archaeal protein translation, i.e. translating selected mRNA into a polypeptide chain, were less successful. Now, towards a better understanding of translation initiation in Archaea, an *in vitro* translation system for translation of added mRNA has been developed for the haloarchaeon *Haloferax volcanii*.

## Materials and methods

### Materials

Alumina (aluminium oxide), ampicillin, diethylpyrocarbonate (DEPC) and 2-mercaptoethanol were obtained from Sigma (St Louis, MO). ATP, brewer's yeast tRNA, T7 RNA polymerase buffer, CTP, a FastStart high-fidelity PCR system, GTP, RNase-free DNase, RNase inhibitor, T7 RNA polymerase and UTP came from Roche (Mannheim, Germany). Restriction enzymes came from New England Biolabs (Hertfordshire, UK), Fermentas (Burlington, CA) and Takara (Shiga, Japan). DNA markers came from New England Biolabs and Fermentas. Molecular weight markers and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies were purchased from BioRad (Hercules, CA). An ECL-enhanced chemiluminescence kit was obtained from Amersham (Buckingham, UK), as was the Redivue Pro-Mix [<sup>35</sup>S] cell-labelling mix (> 1000 Ci mmol<sup>-1</sup>).

### Preparation of *H. volcanii* cell extract

*Haloferax volcanii* cells were grown in rich medium (Irihimovitch *et al.*, 2003) at 40 °C to the mid-exponential phase (OD<sub>550nm</sub> = 1.2), harvested by centrifugation (9000 g, 10 min), frozen in liquid nitrogen and stored at -80 °C until use. To prepare cell extracts, the frozen cells were treated as described previously (Ring & Eichler, 2004), with slight modifications. Cell lysates were prepared by disrupting the cells (6 g) in a precooled mortar with twice their weight of alumina and 2 vol of extraction buffer on ice. In the case of poly(U) conditions (see below), the cells were

extracted with buffer S (2.5 M KCl, 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 M NH<sub>4</sub>Cl, 60 mM Mg(OAc)<sub>2</sub>, 5 mM 2-mercaptoethanol, 30 mM Tris-HCl, pH 8.0), while for extraction under physiological conditions, buffer P (3.4 M KCl, 100 mM Mg(OAc)<sub>2</sub>, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.6) was used. Next, 1 µg mL<sup>-1</sup> of RNase-free DNase was added and the extract was incubated for 30 min on ice. The alumina and cell debris were removed by centrifugation twice at 30 000 g, for 30 min at 4 °C. The supernatant (S30) was stored in aliquots at -80 °C.

### Gene construction

The *H. volcanii* ribosomal protein L11 and dihydrofolate reductase (DHFR) genes were PCR-amplified using forward primers (L11f and DHFRf, respectively) containing a KpnI site, followed by a tract of the 5'-untranslated region (UTR) lying 60 nucleotides upstream of the start codon of the gene of interest and reverse primers (L11r and DHFRr, respectively) directed at the 3' end of each gene, followed by a BamHI site. In the case of DHFR, the gene was recloned, this time using a forward primer DHFRfΔ directed against a region downstream of the 5'-UTR and beginning with a KpnI site, thereby yielding a leaderless gene. The gene encoding for proteasome-activating nucleotidase A (PanA) was cloned using forward primer PanAf containing a DraIII site and the T7 promoter sequence and reverse primer PanAr directed at the 3' end of the gene, followed by a BamHI site. In the case of RadA, forward primer RadAf was directed against a region at the start of the 60 nucleotide-long 5'-UTR, beginning with a KpnI site, while reverse primer RadAr was directed at the 3' end of the gene, followed by a sequence encoding a stretch of six histidine residues and a BamHI site. In these PCR amplifications, *H. volcanii* genomic DNA served as a template. To prepare DNA encoding the *Clostridium thermocellum* cellulosome cellulose-binding domain (CBD) designed to yield mRNA bearing a 5'-UTR including an SD motif, forward primer CBDSDf, containing a KpnI site and the SD motif and reverse primer CBDr, directed at the 3' end of the gene followed by a BamHI site, were used. To yield a leaderless version of CBD RNA, the CBD sequence was recloned with forward primer CBDfΔ containing a DraIII site and the T7 promoter sequence, yielding a leaderless gene. In these PCR amplifications, plasmid pWL-CBD-SecY (Irihimovitch *et al.*, 2003) served as the template. The sequences of all primers used are listed in Table 1. Following PCR amplification using a high-fidelity DNA polymerase, the constructs were digested with the appropriate restriction enzymes and inserted into the corresponding sites of cloning vector pBluescript SK(+) (pBS-SK+) to yield plasmids pBS-L11, pBS-DHFR, pBS-ΔL-DHFR, pBS-PanA, pBS-RadA, pBS-SD-CBD and pBS-ΔL-CBD. Before run-off transcription,

**Table 1.** List of primers used in this study

Primer	Sequence
L11f	5'- <u>CCC</u> GGTACCTGCGTAGTTGTCGCACACG -3'
L11r	5'- CCCGGATCCAAGCGGGCCGAGTCGAC -3'
DHFRf	5'- CCCGGTACCGACGGGAGGCGACGCCG -3'
DHFRfΔ	5'- CCCGGTACCGATGGAACCTGCTCTGTGCG -3'
DHFRr	5'- CCCGGATCCCTATCTGGACGACGCCGA -3'
PanAf	5'- GAGCACTACGTG <b>TAATACGACTCACTATAGG</b> CGGATGATG ACCGATACTGTG -3'
PanAr	5'- ATCGGATCCTTACGCGAACGCGCGGGAGAC -3'
RadAf	5'- GAGGGTACCTTTTCACTTTCATCTTGCTGTTAACGAGGG -3
RadAr	5'- CTCGGATCCTTAgtggtggtggtggtggtCTCGGGCTTGAGACCCGC GTCC -3'
CBDSDf	5'- GAGGGTACCGCGTACTAC <b>GGAGGTG</b> ATAATGGCAAATACA CCGGTATC -3'
CBDfΔ	5'- GAGCACACGGT <b>TAATACGACTCACTATAGG</b> ACAATGGCA AATACACCGGTATCAGG -3'
CBDr	5'- ATCGGATCCCTATACTACTGCCACCGGGTTC -3'

Restriction sites used for the cloning are underlined. The T7 promoter sequence is shown in bold, while SD motifs are shown in bold italics. The polyhistidine-encoding sequence is shown in lowercase.

the plasmids were linearized by digestion with NotI, XbaI or SacI.

### ***In vitro* transcription**

For run-off transcription, constructs (3–5 µg) were linearized with the appropriate restriction enzymes and incubated for 2 h at 37 °C in a mixture containing 1 × T7 RNA polymerase buffer, 1–2 mM NTPs, 20 U of RNase inhibitor and 40 U of T7 RNA polymerase. Forty units of RNase-free DNase were added, followed by an additional incubation at 37 °C for 1 h. The transcribed mRNA was purified by phenol–chloroform extraction as follows: 1 vol of phenol, pH 5.5–6.5, was added to the mRNA-containing samples, vortexed and centrifuged at 15 000 g for 2 min, at 4 °C. The aqueous phase containing the mRNA was transferred to a new tube and extracted an additional two times: once with 0.5 vol of phenol and 0.5 vol of chloroform:isoamylalcohol (99:1) and then with 1 vol of chloroform:isoamylalcohol. Next, 1 vol of 4 M ammonium acetate and 4 vol of 95% EtOH were added in order to precipitate the mRNA. After a 30-min incubation at –80 °C, the samples were centrifuged at 15 000 g for 30 min at 4 °C, the supernatant was removed and the tubes were left to dry. The mRNA pellets were resuspended in DEPC-treated DDW and stored at –20 °C. Before translation, the size and integrity of the transcribed mRNAs were verified by electrophoresis on denaturing agarose gels.

### ***In vitro* translation**

For *in vitro* translation of the total *H. volcanii* mRNA pool, 100 µL (240 µg) of the *H. volcanii* S30 extraction prepared under either poly(U) or physiological conditions were incubated for 10 min at 40 °C, to allow decharging of the ribosomes, thereby reducing the contribution of translated

endogenous mRNAs during detection of newly synthesized polypeptides as much as possible. In the case of poly(U) conditions, the preincubated S30 fraction was combined, in a final volume of 500 µL, with 0.08 g KCl, 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 M NH<sub>4</sub>Cl, 30 mM Mg(OAc)<sub>2</sub>, 30 mM Tris-HCl, pH 8.0, 6 mM ATP, 2 mM GTP, 7 mM phosphoenolpyruvate, 20 µM of all amino acids except methionine and cysteine, 0.1 µg µL<sup>-1</sup> bulk brewer's yeast tRNA and [<sup>35</sup>S] cysteine/methionine (14 µCi mL<sup>-1</sup>). In the case of physiological conditions, the preincubated S30 fraction was mixed, in a final volume of 500 µL, with 0.1 g KCl, 0.4 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 M NH<sub>4</sub>Cl, 50 mM Mg(OAc)<sub>2</sub>, 20 mM Tris-HCl, pH 7.6, 6 mM ATP, 2 mM GTP, 7 mM phosphoenolpyruvate, 20 µM of all amino acids except methionine and cysteine, 0.1 µg µL<sup>-1</sup> bulk brewer's yeast tRNA and [<sup>35</sup>S] cysteine/methionine (14 µCi mL<sup>-1</sup>). Both sets of reaction mixtures were incubated at 40 °C, with 50 µL aliquots being removed at increasing times. The level of radioactive label-incorporating protein present in each aliquot was determined by scintillation counting in a β-counter and by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography using Kodak Bio-Max XAR film.

For individual mRNAs, the same translation mixtures as above were prepared, except that reactions were performed in 50 µL volumes. Accordingly, 10-fold less KCl was added to each reaction. To each reaction, 6–8 µg of transcribed mRNA was added. At the end of a 90-min incubation at 40 °C, the samples were precipitated with trichloroacetic acid and subjected to 15% SDS-PAGE, followed by fluorography and/or immunoblotting. In control experiments, no exogenous mRNA was added. Finally, in some cases, microcococcus nuclease was added to the translation reactions to eliminate any endogenous mRNA, according to a protocol described previously (Gropp & Oesterhelt, 1989).

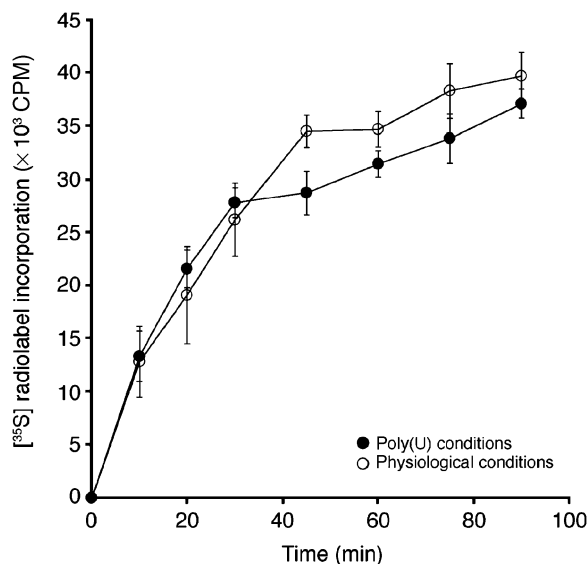
## Immunoblotting

Immunoblotting was performed using antibodies raised against *H. volcanii* DHFR (obtained from Moshe Mevarech, Tel Aviv University; Ortenberg *et al.*, 2000) or PanA (obtained from Julie Maupin-Furlow, University of Florida; Reuter *et al.*, 2004), against the *C. thermocellum* CBD (obtained from Yuval Shoham, Technion Israel Technology Institute) or against polyhistidine tags (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was detected using goat anti-rabbit HRP-conjugated antibodies and ECL-enhanced chemiluminescence.

## Results

### *In vitro* translation of total RNA by *H. volcanii* ribosomes

Towards developing a system derived from *H. volcanii* components capable of translating individual mRNAs, efforts were first directed at determining the optimal conditions for the *in vitro* translation of the pool of total *H. volcanii* RNA. In previous studies in which *in vitro* haloarchaeal poly(U)-dependent poly(Phe) synthesis systems were developed, it was reported that optimal translation was realized using a set of artificial conditions, referred to hereafter as poly(U) conditions, in which both KCl and  $\text{NH}_4^+$  ions are present at high concentrations (Saruyama

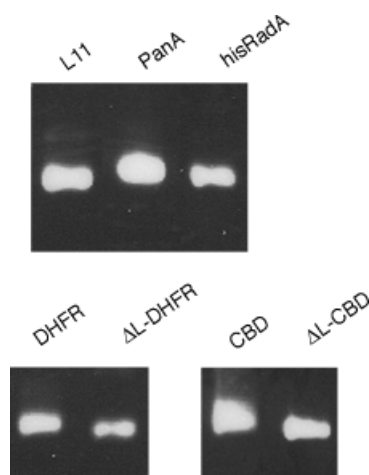


**Fig. 1.** *In vitro* translation of total *Haloferax volcanii* RNA. Total *H. volcanii* RNA was translated as described in 'Materials and methods' under either poly(U) or physiological conditions. Aliquots (50  $\mu\text{L}$ ) were removed at the indicated times and 10  $\mu\text{L}$  samples were subjected to  $\beta$ -counting. Values obtained using poly(U) conditions are depicted by full circles, while values obtained with physiological conditions are depicted by open circles. Each value represents the average of three separate experiments  $\pm$  SEM.

& Nierhaus, 1985; Sanz *et al.*, 1988). In the present study, protein translation was readily achieved when total *H. volcanii* RNA was incubated with an *H. volcanii* ribosome-containing cell lysate prepared under poly(U) conditions, in the presence of 2.5 M KCl and 2 M  $\text{NH}_4^+$  ions, as gauged by [ $^{35}\text{S}$ ]cysteine/methionine incorporation into newly synthesized proteins via scintillation counting (Fig. 1). Similar results were also obtained when *in vitro* translation of total *H. volcanii* RNA was performed using ribosomes maintained under physiological conditions, i.e. where  $\text{K}^+$  ions are found at elevated levels, reminiscent of the situation in the haloarchaeal cytoplasm, but  $\text{NH}_4^+$  ions, found at enhanced levels under the non-natural poly(U) incubation conditions are not.

### *In vitro* translation of individual *H. volcanii* mRNAs presenting SD motifs

The ability of the reconstituted system to translate individual mRNAs was next considered. For this purpose, mRNA encoding selected *H. volcanii* proteins (see below) was transcribed *in vitro* as described in 'Materials and methods' (Fig. 2). Translation of the transcribed messages was then attempted using *H. volcanii* ribosomes under both poly(U) and physiological conditions. Any *in vitro* translated product was next detected by either one of two approaches. In the first approach, the appearance of a novel or enhanced [ $^{35}\text{S}$ ] cysteine/methionine-incorporating polypeptide of the expected molecular weight was followed following SDS-



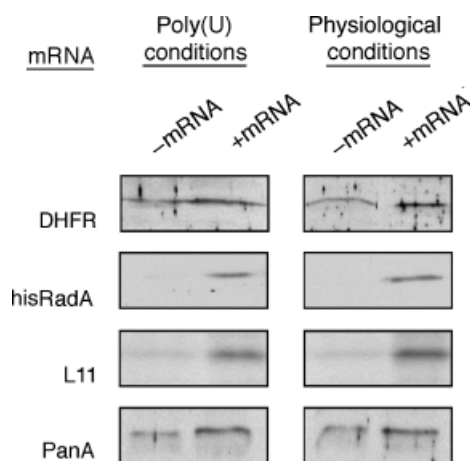
**Fig. 2.** *In vitro* transcription of selected mRNAs. After *in vitro* transcription of the individual constructs described in 'Materials and methods', mRNAs were purified by phenol-chloroform extraction. An aliquot of each transcript was loaded onto a 2% denaturing agarose gel and subjected to electrophoresis. The upper panel shows the migration of aliquots of L11, PanA and hisRadA mRNA. The lower panels (from left to right) show the migration of mRNA encoding DHFR and *Clostridium thermocellum* CBD, in each case either containing an SD motif (right lane) or lacking the leader sequence (left lane).

**Table 2.** List of mRNAs added to the *Haloferax volcanii* *in vitro* translation system

mRNA	Translation initiation sequence				SD sequence
	5'-UTR	'Strong' SD*	'Weak' SD	No SD	
DHFR	+	+			GGGAGUGA
L11	+		+		CACGGUGU
RadA-his	+		+		CGGAACGA
PanA	-			+	-
ΔL-DHFR	-			+	-
CBD	-			+	-
SD-CBD	+	+			GGAGGUGA

\*Shine-Dalgarno motif.

+, present; -, absent.



**Fig. 3.** *In vitro* translation of selected *Haloferax volcanii* mRNAs. Translation reactions were performed under either poly(U) or physiological conditions (left and right columns, respectively), each time either in the absence (left lane) or presence (right lane) of added *in vitro* transcribed mRNA. Translation products were detected either by following [<sup>35</sup>S] radiolabel incorporation via SDS-PAGE and fluorography (L11) or by immunoblotting using antibodies (against DHFR, PanA or the polyhistidine tag of hisRadA). Equivalent aliquots of the reactions either lacking or containing externally added mRNA were analysed in each case.

PAGE and fluorography. This approach was not, however, applicable in all cases, given the high level of metabolically radiolabelled nascent polypeptides with apparent molecular weights above 30 kDa encoded by endogenous mRNA found within the ribosome-containing cell lysate. Such mRNA yielded a background level of protein translation that obscured the appearance of products above 30 kDa putatively derived from any added mRNA. Moreover, in contrast to earlier studies (Gropp & Oesterhelt, 1989), addition of microcococcus nuclease, designed to remove endogenous mRNA, had no effect on the level of this background translation. As such, the translation of some added mRNAs could only be followed using antibodies raised against the encoded protein or an introduced polyhistidine tag. In some

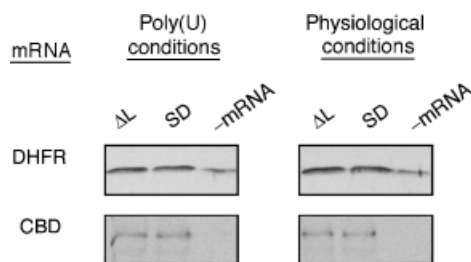
instances, both detection methods were used. Finally, control experiments were performed in each case in which no *in vitro* transcribed mRNA was included in the reaction mixture.

Initially, translation of mRNAs preceded by a 5'-UTR that includes a 'strong' SD motif, i.e. a sequence in which at least five nucleotides of the eight nucleotide-long GGAGGUGA *H. volcanii* SD motif (Sartorius-Neef & Pfeifer, 2004) is present, such as *H. volcanii* DHFR mRNA, was attempted (Table 2). When added to the *in vitro* translation system, enhanced DHFR translation was observed relative to the background, as revealed by immunoblotting using anti-DHFR antibodies (Fig. 3). Densitometric quantification confirmed these observations, showing that 1.5-fold more DHFR ( $1.5 \pm 0.2$ ,  $n = 3$ ) appeared when translation was conducted under poly(U) conditions and twofold more DHFR ( $2.29 \pm 0.1$ ,  $n = 4$ ) appeared when translation was conducted under physiological conditions, relative to what was measured in the background signal, when no exogenous DHFR mRNA was added.

Efforts were next directed at translating those messengers presenting 'weaker' SD initiation motifs, namely where only four nucleotides of the GGAGGUGA SD motif are present (Table 2). The mRNA encoding RadA, a protein involved in *H. volcanii* DNA repair (Woods & Dyall-Smith, 1997), is one such sequence, containing a 'weak' SD motif (CGGAACGA) situated six nucleotides upstream of the first AUG triplet. The addition of mRNA directing the synthesis of polyhistidine-tagged RadA to the *in vitro* translation system under either set of experimental conditions led to the appearance of a novel 38 kDa protein band recognized by antibodies aimed at the polyhistidine tag introduced at the 3'-end of the encoding gene (Fig. 3). Likewise, successful translation of the *H. volcanii* ribosomal large subunit L11 protein component, also encoded by mRNA presenting a 'weak' SD motif (CACGGUGU) lying eight nucleotides upstream of the AUG start codon, was achieved, as reflected by the greatly enhanced level of a [<sup>35</sup>S]radiolabel-incorporating 20 kDa protein band, relative to the background signal obtained when *in vitro* transcribed L11 mRNA was not added to the reaction mixture (densitometrically measured as a  $3.8 \pm 0.3$  increase under poly(U) conditions and as a  $3.3 \pm 0.6$  increase under physiological conditions, with the experiment repeated twice for each set of conditions).

### ***In vitro* translation of leaderless vs. SD motif-bearing mRNA**

Given the predominance of leaderless mRNAs in halophilic archaea like *Halobacterium* sp. NRC-1 and *Haloarcula marismortui* (and indeed in other archaeal species) (Ma et al., 2002; Torarinsson et al., 2005; Chang et al., 2006), the *in vitro* translation system developed here was tested for its



**Fig. 4.** *In vitro* translation of selected mRNAs both presenting SD motifs or lacking leader sequences. *In vitro* transcribed mRNA encoding *Haloferax volcanii* DHFR (top panels) or *Clostridium thermocellum* CBD (lower panels) were translated under poly(U) or physiological conditions (left and right columns, respectively). In each case, the mRNA either lacked a leader sequence (left lanes) or contained a 5'-UTR including an SD motif (middle lanes). Alternatively, no *in vitro* transcribed mRNA was added to the translation reaction in control reactions (right lanes). Equivalent aliquots of the reactions either lacking or containing externally added mRNA were analysed in each case. Translation products were detected via SDS-PAGE and immunoblotting, using appropriate antibodies.

ability to translate mRNA lacking leader sequences, using either poly(U) or physiological conditions. The mRNA encoding PanA, a 45 kDa protein involved in *H. volcanii* protein degradation (Reuter *et al.*, 2004), presents a stretch of noncoding mRNA only three nucleotides long upstream to the AUG start codon. Nonetheless, the absence of both an SD motif and a leader sequence did not prevent *in vitro* translation of PanA mRNA, as revealed by immunostaining using antibodies raised against the protein (Reuter *et al.*, 2004), with  $1.9 \pm 0.2$  ( $n=2$ ) more protein appearing upon addition of mRNA encoding the protein under poly(U) conditions and  $1.7 \pm 0.1$  ( $n=2$ ) more protein appearing under physiological conditions (Fig. 3).

As both SD motif-bearing and leaderless mRNA are translated by the *in vitro* translation system developed in this study, regardless of which set of incubation conditions was used, experiments were conducted to determine which mode of translation initiation is preferred by the *H. volcanii* system. Accordingly, mRNA encoding DHFR, either bearing its native upstream untranslated region, including the 'strong' SD motif, or lacking its leader sequence, was added to the *in vitro* translation system. Both versions of DHFR mRNA were translated to comparable extents, as detected by immunoblotting using anti-DHFR antibodies (Fig. 4) or by fluorography following translation in the presence of [<sup>35</sup>S] cysteine/methionine (not shown). Again, a background level of DHFR derived from endogenous messenger was obtained in the absence of added mRNA. Densitometric analysis revealed that similar amounts of DHFR ( $1.0 \pm 0.2$ ,  $n=3$ ) were produced from mRNA containing or lacking a leader sequence with its SD motif when translation was performed under poly(U) conditions. When the results of the same reactions performed under physiological conditions were

densitometrically quantified, it was determined that DHFR mRNA containing its SD motif-bearing leader directed the translation of  $1.3 \pm 0.4$  ( $n=4$ ) times more product than did DHFR mRNA lacking a leader sequence.

Finally, the ability of the *H. volcanii in vitro* translation system to translate nonarchaeal mRNA was tested. In these experiments, the translation of different versions of *in vitro* transcribed mRNA encoding a bacterial protein, namely the CBD of the *C. thermocellum* cellulosome (Morag *et al.*, 1995), was attempted. In the first version of mRNA considered, the CBD-encoding sequence was preceded by an introduced leader sequence including a 14-nucleotide sequence comprising a 'strong' SD motif (i.e. that of *H. volcanii* L1 mRNA), together with the three nucleotides flanking each end of the motif. In a second mRNA, no leader sequence was present, such that only the sequence encoding the CBD moiety was transcribed. As shown in Fig. 4, CBD mRNA either preceded by an SD motif-bearing leader sequence or instead lacking a leader sequence was translated by the *H. volcanii in vitro* translation system under both sets of experimental conditions, with translation dictated by mRNA preceded by an SD motif-bearing leader sequence producing up to 25% more product than messenger lacking a leader, much as seen with DHFR mRNA, above.

## Discussion

Although members of both the bacterial and eukaryal domains of life express proteins using similar machineries, it is generally accepted that Bacteria rely on a distinct mode of translation initiation from that found in Eukarya. In Bacteria, mRNAs are generally polycistronic and endowed with 5'-UTRs. Most of the ORFs are also preceded by an SD motif, responsible for binding a complementary sequence at the 3'-end of 16S rRNA gene. Three initiation factors, IF1, IF2 and IF3, mediate the interaction between the ribosome and the mRNA start site (Kozak, 1983; Gualerzi & Pon, 1990). By contrast, eukaryal translation initiation does not involve direct ribosome-mRNA contact, but rather involves binding of the small ribosomal subunit, via a number of accessory proteins, to the capped 5'-end of the messenger. This ribosome-based complex then 'slides' along the mRNA until the first AUG codon is encountered and translation can begin (Kozak, 1989, 2002). However, when the third domain of life, the Archaea (Woese & Fox, 1977), is taken into account, the clear distinction between prokaryal and eukaryal translation initiation no longer holds true. Like Bacteria, Archaea are endowed with polycistronic mRNAs in which the individual cistrons may be preceded by SD motifs (Dennis, 1997; Bell & Jackson, 1998; Londei, 2005). On the other hand, Archaea also possess a major proportion of mRNAs that lack a 5'-UTR entirely, or almost so (Ma *et al.*, 2002; Torarinsson *et al.*, 2005; Chang *et al.*, 2006).

While such leaderless mRNAs are found in all three domains of life (O'Connor *et al.*, 1999; Moll *et al.*, 2002), they are especially abundant in Archaea, accounting for 50% of all mRNAs in some species (Torarinsson *et al.*, 2005). The presence of leaderless mRNA has led to the proposal that Archaea rely on two distinct translation initiation pathways (Tolstrup *et al.*, 2000; Benelli *et al.*, 2003). Finally, Archaea contain over 10 genes encoding homologues of eukaryal initiation factors, thus displaying a complement of initiation factors far more complex than that found in Bacteria. Thus, unravelling the features of translational initiation in Archaea is important for understanding the evolution of this stage of protein translation.

To date, both *in vivo* and *in vitro* approaches have been employed to address translation initiation in Archaea, largely using the thermoacidophile *S. solfataricus* and haloarchaeal strains as model systems. *In vivo* studies have confirmed that SD motifs are indeed recognized by Archaea (Benelli *et al.*, 2003; Sartorius-Neef & Pfeifer, 2004). However, discrepancies exist between the various models in terms of the dependence of translation initiation on the SD motifs present in archaeal mRNA 5'-UTRs. Using an *in vitro* translation system derived from the thermoacidophile *S. solfataricus* (Condo *et al.*, 1999), it was demonstrated that mutation of SD motifs completely blocked protein translation. In fact, *S. solfataricus* ribosomes are unable to bind productively to leadered mRNAs lacking SD motifs *in vitro* (Benelli *et al.*, 2003). By contrast, it was subsequently reported that protein biosynthesis was only reduced upon partial or even complete mutation of SD motifs in *Halobacterium salinarum* reporter mRNAs expressed in *H. volcanii* (Sartorius-Neef & Pfeifer, 2004). Studies on *S. solfataricus* have also shown that the small ribosomal subunit does not directly bind to the initiating AUG codon of leaderless mRNAs, but rather requires an initiator tRNA intermediate (Benelli *et al.*, 2003). The use of haloarchaeal mutant strains showed, furthermore, that codons other than AUG, encoding amino acids other than methionine, can be used for initiating protein synthesis in Archaea (Srinivasan *et al.*, 2006), in seeming distinction from the translation of leaderless bacterial mRNAs (Van Etten & Janssen, 1998). Thus, to reconcile apparent differences suggested from *in vivo* experiments on haloarchaea and using *S. solfataricus* cells or the reconstituted system, to expand upon previous findings as well as to obtain a novel insight into archaeal translation initiation, an *in vitro* translation system derived from haloarchaeal components has been developed in this study.

Using the *H. volcanii* *in vitro* translation system, it was demonstrated that halophilic ribosomes are indeed able to translate both leadered mRNAs endowed with either 'strong' or 'weak' SD motifs as well as leaderless mRNAs. Interestingly, the comparable levels of DHFR mRNA expression, either initiated through its SD motif or using a leaderless

transcript, suggest that the two translation initiation pathways in haloarchaea are almost equally effective using transcripts of native genes. This contrasts with findings in the thermoacidophile *S. solfataricus*, where the SD motif-primed translation of a leadered reporter mRNA was considerably more efficient than that of the same mRNA rendered leaderless by the deletion of its 5'-UTR (Condo *et al.*, 1999). On the other hand, the results of the present study, obtained using an *in vitro* translation system, are in agreement with the findings of Sartorius-Neef & Pfeifer (2004). In that *in vivo* study, it was shown that even extensive mutation of the SD motifs of mRNAs derived from halophilic genes did not substantially impair their translation. On the whole, the results of both studies suggest that halophilic ribosomes may, in general, possess a high binding affinity for mRNAs and yet are less discriminatory with respect to the relative strengths of the different ribosome-binding signals.

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