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The gene of an archaeal α-L-fucosidase is expressed by translational frameshifting

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ABSTRACT

The standard rules of genetic translational decoding are altered in specific genes by different events that are globally termed recoding. In Archaea recoding has been unequivocally determined so far only for termination codon readthrough events. We study here the mechanism of expression of a gene encoding for a α-L-fucosidase from the archaeon *Sulfolobus solfataricus* (*fucA1*), which is split in two open reading frames separated by a −1 frameshifting. The expression in *Escherichia coli* of the wild-type split gene led to the production by frameshifting of full-length polypeptides with an efficiency of 5%. Mutations in the regulatory site where the shift takes place demonstrate that the expression *in vivo* occurs in a programmed way. Further, we identify a full-length product of *fucA1* in *S.solfataricus* extracts, which translate this gene *in vitro* by following programmed −1 frameshifting. This is the first experimental demonstration that this kind of recoding is present in Archaea.

INTRODUCTION

Translation is optimally accurate and the correspondence between the nucleotide and the protein sequences are often considered as an immutable dogma. However, the genetic code is not quite universal: in certain organelles and in a small number of organisms the meaning of different codons has been reassigned and all the mRNAs are decoded accordingly. More surprisingly, the standard rules of genetic decoding are altered in specific genes by different events that are globally termed recoding (1). In all cases, translational recoding occurs in competition with normal decoding, with a proportion of the ribosomes not obeying to the 'universal' rules. Translational recoding has been identified in both prokaryotes and eukaryotes. It has crucial roles in the regulation of gene expression and includes stop codon readthrough, ribosome hopping and ±1 programmed frameshifting [for reviews see (2–4)].

In stop codon readthrough a stop codon is decoded by a tRNA carrying an unusual amino acid rather than a translational release factor. Specific stimulatory elements downstream to the stop codon regulate this process (5). Hopping, in which the ribosome stops translation in a particular site of the mRNA and re-start few nucleotides downstream, is a rare event and it has been studied in detail only in the bacteriophage T4 (6). In programmed frameshifting, ribosomes are induced to shift to an alternative, overlapping reading frame 1 nt 3′-wards (+1 frameshifting) or 5′-wards (−1 frameshifting) of the mRNA. This process is regulated and its frequency varies in different genes. The ±1 programmed frameshifting has been studied extensively in viruses, retrotransposons and insertion elements for which many cases are documented (7–9). Instead, this phenomenon is by far less common in cellular genes. A single case of programmed +1 frameshifting is known in prokaryotes (10,11) while in eukaryotes, including humans, several genes regulated by this recoding event have been described previously [(4) and references therein]. Compared to +1 frameshifting, −1 frameshifting is less widespread with only two examples in prokaryotes (12–14) and few others in eukaryotes (15–17).

The programmed −1 frameshifting is triggered by several elements in the mRNA. The slippery sequence, showing the X-XXY-YYZ motif, in which X can be any base, Y is usually A or U, and Z is any base but G, has the function of favouring the tRNA misalignment and it is the site where the shift takes place (3,18). Frameshifting could be further stimulated by other elements flanking the slippery sequence: a codon for a low-abundance tRNA, a stop codon, a Shine–Dalgarno sequence and an mRNA secondary structure. It has been

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reported that these elements, alone or in combination, enhance frameshifting by pausing the translating ribosome on the slippery sequence (4,18).

Noticeably, known cases of recoding in Archaea [recently reviewed in (19)] are limited to termination codon readthrough events that regulate the incorporation of the 21st and 22nd amino acids selenocysteine and pyrrolysine, respectively (20–23).

No archaeal genes regulated by translational programmed frameshifting and ribosome hopping have been identified experimentally so far; therefore, if compared with the others domains of life, the study of translational recoding in Archaea is still at its dawn.

We showed that the α-L-fucosidase gene from the crenarchaeon *Sulfolobus solfataricus* is putatively expressed by programmed −1 frameshifting (24). This gene, named *fucA1*, is organized in the open reading frames (ORFs) SSO11867 and SSO3060 of 81 and 426 amino acids, respectively, which are separated by a −1 frameshifting in a 40 base overlap (Figure 1A). We have reported previously that the region of overlap between the two ORFs had the characteristic features of the genes expressed by programmed −1 frameshifting including a slippery heptanucleotide A-AAA-AAT (codons are shown in the zero frame) flanked by a putative stem–loop and the rare codons CAC (Figure 1A) resembling the prokaryotic stem–loops/hairpins and the Shine–Dalgarno-like sites (24). We showed that the frameshifting, obtained by mutating by site-directed mutagenesis the *fucA1* gene exactly in the position predicted from the slippery site, produced a full-length gene, named *fucA1*^{A}, encoding for a polypeptide of 495 amino acids (Figure 1B). This mutant gene expressed in *Escherichia coli* a fully functional α-L-fucosidase, named Ssα-fuc, which was thermophilic, thermostable and had an unusual nonameric structure (24,25). More recently, we determined the reaction mechanism and the function of the residues of the active site of the mutant enzyme (26,27).

The functionality of the product of the mutant gene *fucA1*^{A} does not provide direct experimental evidence that programmed −1 frameshifting occurs *in vivo* and in *S.solfataricus*. To address these issues, we report here the study of the expression of the wild-type split gene *fucA1* and of its mutants in the slippery sequence. We demonstrate here that *fucA1* is expressed by programmed −1 frameshifting in both *E.coli* and *S.solfataricus*. This is the first experimental demonstration that this kind of recoding is present in the Archaea domain of life. The relevance of programmed −1 frameshifting in Archaea is also discussed.

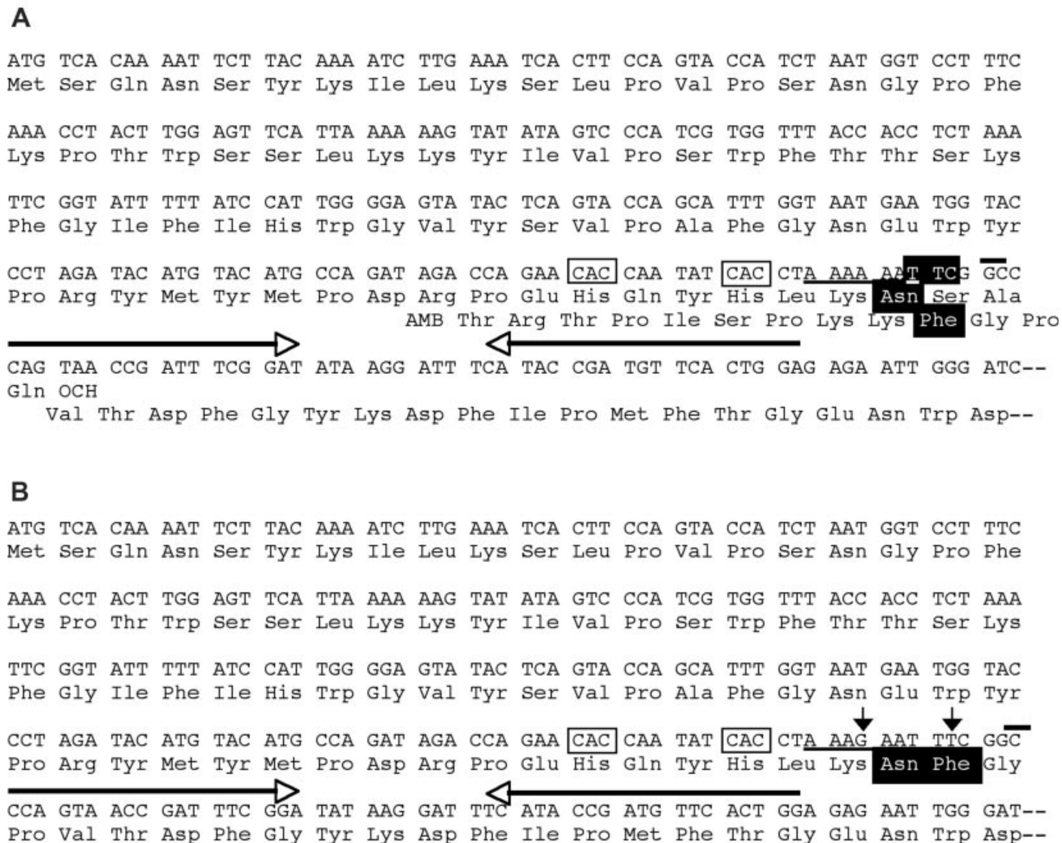

Figure 1. The α-fucosidase gene. (**A**) Region of overlap in the wild-type split *fucA1* gene. The N-terminal SSO11867 ORF is in the zero frame, the C-terminal SSO3060 ORF, for which only a fragment is shown, is in the −1 frame. The slippery heptameric sequence is underlined; the rare codons are boxed and the arrows indicate the stems of the putative mRNA secondary structure. The amino acids involved in the programmed −1 frameshifting and the first codon translated after this event in the −1 frame are shadowed. (**B**) Fragment of the full-length mutant *fucA1*^{A} gene. The small arrows indicate the mutated nucleotides.

MATERIALS AND METHODS

Analysis of the α-fucosidase expression

S.solfataricus cells were grown, and cell extracts obtained, as described previously (24,28).

The expression in the *E.coli* strain BL21(RB791) of the wild-type gene *fucA1* and of the mutant genes *fucA1*^{A} [previously named FrameFuc in (24)], *fucA1*^{B}, *fucA1*^{sm} and *fucA1*^{tm} as fusions of glutathione *S*-transferase (GST) and the purification of the recombinant proteins were performed as reported previously (23). The nomenclature used in this paper for the different α-fucosidase genes is listed in Table 1.

For the western blot studies, equal amounts of *E.coli* cultures expressing the wild-type and mutant *fucA1* genes, normalized for the OD_{600}, were resuspended in SDS–PAGE loading buffer containing 0.03 M Tris–HCl buffer, pH 6.8, 3% SDS (w/v), 6.7% glycerol (w/v), 6.7% 2-mercaptoethanol (w/v) and 0.002% blue bromophenol (w/v). The samples were incubated at 100°C for 5 min (unless otherwise indicated) and were directly loaded on to the gel. Western blot analyses were performed by blotting SDS–PAGEs of the concentrations indicated on Hybond-P polyvinylidenfluorid filters (Amersham Biosciences, Uppsala, Sweden); polyclonal anti-Ssα-fuc antibodies from rabbit (PRIMM, Milan, Italy) and anti-GST antibodies (Amersham Biosciences) were diluted 1:5000 and 1:40 000, respectively. The filters were washed and incubated with the ImmunoPure anti-rabbit IgG antibody conjugated with the horseradish peroxidase (HRP) from Pierce Biotechnology (Rockford, IL, USA). Filters were developed with the ECL-plus Western Blotting Detection system (Amersham Biosciences) by following the manufacturer's indications. The molecular weight markers used in the western blot analyses were the ECL streptavidin–HRP conjugate (Amersham Biosciences).

The protein concentration of the samples was measured with the method of Bradford (29) and the amounts of sample loaded on to the SDS–PAGEs are those indicated. The quantification of the bands identified by western blot was performed by using the program Quantity One 4.4.0 in a ChemiDoc EQ System (Bio-Rad, Hercules, CA, USA) with the volume analysis tool. The frameshifting efficiency was calculated as the ratio of the intensity of the bands of the frameshifted product/frameshifted product + termination product.

The mutants in the slippery sequence of the wild-type gene *fucA1* were prepared by site-directed mutagenesis from the vector pGEX-11867/3060, described previously (24,27). The synthetic oligonucleotides used (PRIMM) were the following: FucA1sm-rev, 5′-TTTAGGTGATATTGGTGTTCTGGTCTATCT-3′; FucA1sm-fwd, 5′-GAACACCAATATCACCTAAA<u>G</u>AATTCGGCCCA-3′; FucA1tm-rev, 5′-AGGTGATATTGG<u>T</u>GTTCTGGTCTATCTGGC-3′; FucA1tm-fwd, 5′-CCAGAACACCAATATCACCT<u>C</u>AA<u>G</u>AA<u>C</u>TCGGCCCAGT-3′, where the mismatched nucleotides in the mutagenic primers are underlined. Direct sequencing identified the plasmids containing the desired mutations and the mutant genes, named *fucA1*^{sm} and *fucA1*^{tm}, were completely re-sequenced.

Expression and characterization of Ssα-fuc^B

The mutant Ssα-fuc^B was prepared by site-directed mutagenesis from the vector pGEX-11867/3060, by using the same site-directed mutagenesis kit described above. The synthetic oligonucleotides used were FucA1sm-rev (described above) and the following mutagenic oligonucleotide: Fuc-B, 5′-GAACACCAATATCACCTAAA<u>G</u>AA<u>G</u>TTCGGCCCAGT-3′, where the mismatched nucleotides are underlined. Direct sequencing identified the plasmid containing the desired mutations and the mutant gene, named *fucA1*^{B}, was completely re-sequenced. The enzymatic characterization of Ssα-fuc^B was performed as described previously (24,27).

Mass spectrometry experiments

Samples of the proteins expressed in *E.coli* from the wild-type gene *fucA1* and the mutants *fucA1*^{A} and *fucA1*^{sm}, purified as described, were fractionated on an SDS–PAGE. Protein bands were excised from the gel, washed in 50 mM ammonium bicarbonate, pH 8.0, in 50% acetonitrile, reduced with 10 mM DTT at 56°C for 45 min and alkylated with 55 mM iodoacetamide for 30 min at room temperature in the dark. The gel pieces were washed several times with the buffer, resuspended in 50 mM ammonium bicarbonate and incubated with 100 ng of trypsin for 2 h at 4°C and overnight at 37°C. The supernatant containing peptides was analysed by MALDIMS on an Applied Biosystem Voyager DE-PRO mass spectrometer using α-cyano-4-hydroxycynnamic acid as matrix. Mass calibration was performed by using the standard mixture provided by manufacturer.

Liquid chromatography online tandem mass spectrometry (LCMSMS) analyses were performed on a Q-TOF hybrid mass spectrometer (Micromass, Waters, Milford, MA, USA) coupled with a CapLC capillary chromatographic system (Waters). Peptide ions were selected in the collision cell and fragmented. Analysis of the daughter ion spectra led to the reconstruction of peptide sequences.

Experiments of translation *in vitro*

Genomic DNA from *S.solfataricus* P2 strain was prepared as described previously (24). A DNA fragment of 1538 nt containing the complete *fucA1* gene, was prepared by PCR, by using the following synthetic oligonucleotides (Genenco, Florence, Italy): FucA1-fwd, 5′-CTGGAG*GCGCGC***TAATACGACTCACTA<u>TAGG</u>**<u>TCAGTTAAATGTCACAAAATTCT</u>-3′; FucA1-rev, 5′-GACTTG*GCGCGC*<u>CTATCTATAATCTAGGATAACCCTTAT</u>-3′, in which the sequence corresponding to the genome of *S.solfataricus* is underlined. In the FucA1-fwd primer, the sequence of the promoter of the T7 RNA polymerase is in boldface and the sequence of the BssHII site is shown in italics. The PCR amplification was performed as described previously (24) and the amplification products were cloned in the BssHII site of the plasmid pBluescript II KS+. The *fucA1* gene was completely re-sequenced to check if undesired mutations were introduced by PCR and the recombinant vector obtained, named pBlu-FucA1, was used for translation *in vitro* experiments.

The plasmids expressing the mutant genes *fucA1*^{A}, *fucA1*^{sm} and *fucA1*^{tm} for experiments of translation *in vitro* were prepared by substituting the KpnI–NcoI wild-type fragment, containing the slippery site, with those isolated from the mutants. To check that the resulting plasmids had the correct sequence, the mutant genes were completely re-sequenced.

The mRNAs encoding wild-type *fucA1* and its various mutants were obtained by *in vitro* run-off transcription. About 2 μg of each plasmid was linearized with BssHII and incubated with 50 U of T7 RNA polymerase for 1 h 30 min at 37°C. The transcription mixtures were then treated with 10 U of DNAseI (RNAse free) for 30 min. The transcribed RNAs were recovered by extracting the samples twice with phenol (pH 4.7) and once with phenol/chloroform 1:1 followed by precipitation with ethanol. The mRNAs were resuspended in DEPC-treated H_2O at the approximate concentration of 0.6 pmol/μl.

In vitro translation assays were performed essentially as described by Condò *et al*. (28). The samples (25 μl final volume) contained 5 μl of *S.solfataricus* cell extract, 10 mM KCl, 20 mM Tris–HCl, pH 7.0, 20 mM Mg acetate, 3 mM ATP, 1 mM GTP, 5 μg of bulk *S.solfataricus* tRNA, 2 μl of [^{35}S]methionine (1200 Ci/mmol at 10 mCi/ml) and ~10 pmol of each mRNA. The mixtures were incubated at 70°C for 45 min. After this time, the synthesized proteins were resolved by electrophoresis 12.5% acrylamide–SDS gels and revealed by autoradiography of the dried gels on an Instant Imager apparatus.

Transcriptional analysis of *fucA1*

Cells of *S.solfataricus*, strain P2, were grown in minimal salts culture media supplemented with yeast extract (0.1%), casamino acids (0.1%), plus glucose (0.1%) (YGM) or sucrose (0.1%) (YSM). The extraction of total RNA was performed as reported previously (24). Total RNA was extensively digested with DNAse (Ambion, Austin, TX, USA) and the absence of DNA was assessed by the lack of PCR amplification with each sets of primers described below. The RT–PCR experiments were performed as reported previously (24) by using the primers described previously that allowed the amplification of a region of 833 nt (positions 1–833, in which the A of the first ATG codon is numbered as one) overlapping the ORFs SSO11867 and SSO3060 (24).

For real-time PCR experiments total cDNA was obtained using the kit Quantitect RT (Qiagen GmbH, Hilden, Germany) from 500 ng of the same preparation of RNA described above. cDNA was then amplified in a Bio-Rad LightCycler using the DyNAmo HS Syber Green qPCR Kit (Finnzymes Oy, Espoo, Finland). Synthetic oligonucleotides (PRIMM) used for the amplification of a region at the 3′ of the ORF SSO3060 were as follows: 5′-Real: 5′-TAAATGGC-GAAGCGATTTTC-3′; 3′-Real: 5′-ATATGCCTTTGTCGC-GGATA-3′ for the gene *fucA1*. 5′-GAATGGGGGTGATA-CTGTCG-3′ and 5′-TTTACAGCCGGGACTACAGG-3′ for the 16S rRNA gene.

For each amplification of the *fucA1* gene was used ~2500-fold more cDNA than that used for the amplification of the 16S rRNA. Controls with no template cDNA were always included. PCR conditions were 15 min at 95°C for initial denaturation, followed by 40 cycles of 10 s at 95°C, 25 s at 56°C and 35 s at 72°C, and a final step of 10 min at 72°C. Product purity was controlled by melting point analysis of setpoints with 0.5°C temperature increase from 72 to 95°C. PCR products were analysed on 2% agarose gels and visualized by ethidium bromide staining.

The expression values of *fucA1* gene were normalized to the values determined for the 16S rRNA gene. Absolute expression levels were calculated as *fucA1*/16S ratio in YSM and YGM cells, respectively. Relative mRNA expression levels (YSM/YGM ratio) were calculated as (*fucA1*/16S ratio in YGM cells)/(*fucA1*/16S ratio in YSM). Each cDNA was used in triplicate for each amplification.

RESULTS

Expression of *fucA1* in *E.coli*

The wild-type *fucA1* gene, expressed in *E.coli* as a GST-fused protein, produced trace amounts of α-fucosidase activity (2.3×10^{-2} units mg^{-1} after removal of GST), suggesting that a programmed −1 frameshifting may occur in *E.coli* (24). The enzyme was then purified by using the GST purification system and analysed by SDS–PAGE revealing a major protein band (Figure 2A). The sample and control bands were excised from the gel, digested *in situ* with trypsin and directly analysed by matrix-assisted laser desorption/ionization mass spectrometry (MALDIMS). As shown in Figure 2B and C, both spectra revealed the occurrence of an identical mass signal at *m*/*z* 1244.6 corresponding to a peptide (Peptide A) encompassing the overlapping region of the two ORFs. This result was confirmed by liquid chromatography online tandem mass spectrometry (LCMSMS) analysis of the peptide mixtures. The fragmentation spectra of the two signals showed the common sequence Asn-Phe-Gly-Pro-Val-Thr-Asp-Phe-Gly-Tyr-Lys in which the amino acid from the ORF SSO11867 is underlined. These results unequivocally demonstrate that the protein containing the Peptide A is produced in *E.coli* by a frameshifting event that occurred exactly within the slippery heptamer predicted from the analysis of the DNA sequence in the region of overlap between the ORFs SSO11867 and SSO3060 (Figure 1A).

Remarkably, the MALDIMS analysis of the products of the wild-type *fucA1* gene revealed the presence of a second Peptide B at *m*/*z* 1258.6 that is absent in the spectra of the Ssα-fuc control protein (Figure 2B and C). The sequence of Peptide B obtained by LCMSMS (Figure 2D) was Lys-Phe-Gly-Pro-Val-Thr-Asp-Phe-Gly-Tyr-Lys. This sequence differs only by one amino acid from Peptide A demonstrating that the interrupted gene *fucA1* expresses in *E.coli* two full-length proteins originated by different −1 frameshifting events. Polypeptide A results from a shift in a site A and it is identical to Ssα-fuc prepared by site-directed mutagenesis (24), suggesting that the expression occurred with the simultaneous P- and A-site slippage. Instead, polypeptide B, named Ssα-fuc^{B}, is generated by frameshifting in a second site B as the result of a single P-site slippage (Figure 2E).

To measure the global efficiency of frameshifting in the two sites of the wild-type gene *fucA1* we analysed the total extracts of *E.coli* by western blot using anti-GST antibodies (Figure 2F). Two bands with marked different electrophoretic mobility were observed: the polypeptide of 78.7 ± 1.1 kDa migrated like GST-Ssα-fuc fusion and was identified as originated from frameshifting in either site A or B of *fucA1*. The protein of 38.1 ± 1.2 kDa, which is not expressed by the mutant gene *fucA1*^{A} (not shown), had an electrophoretic mobility compatible with GST fused to the polypeptide encoded by the

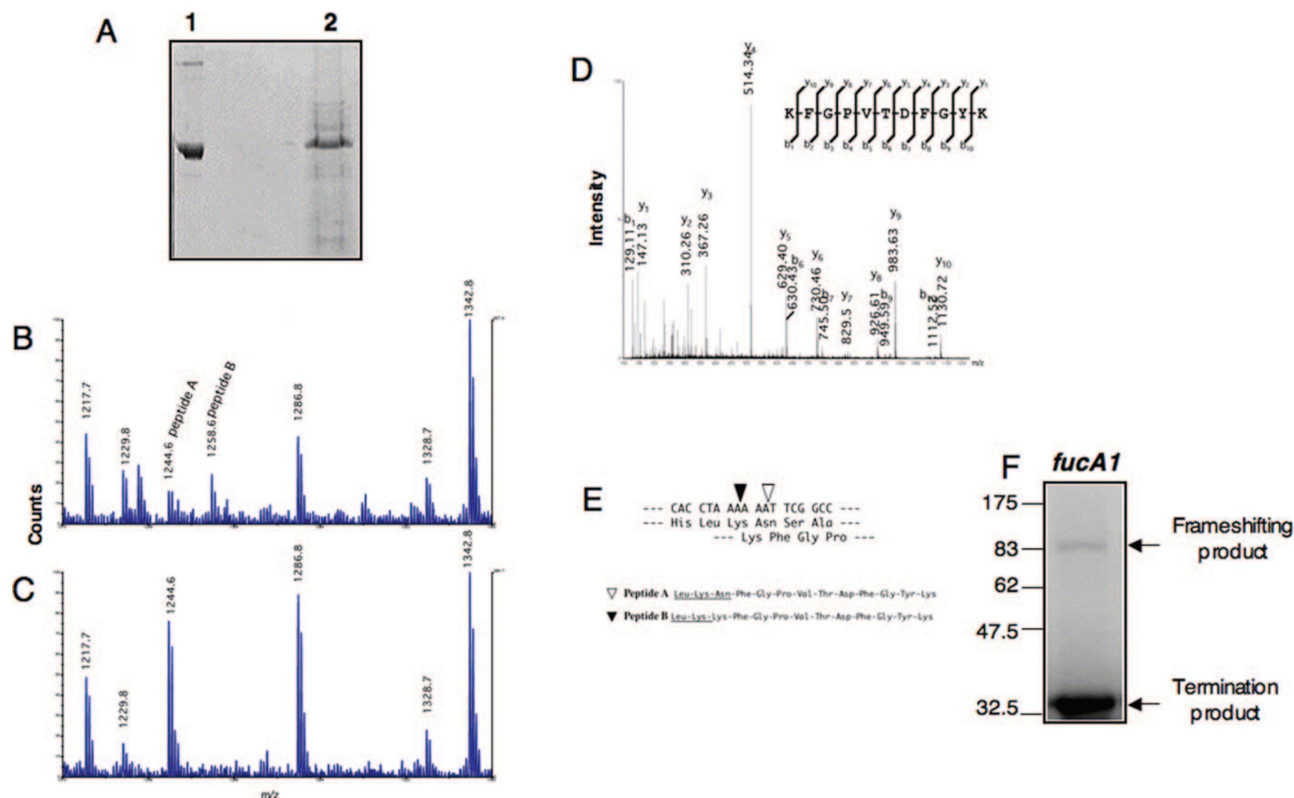

Figure 2. Analysis of the expression of *fucA1* in *E.coli*. (**A**) Coomassie stained 7% SDS–PAGE showing (line 1) the recombinant Ssα-fuc (3 μg) and (line 2) the purified products of the wild-type split *fucA1* gene. The protein concentration of the latter sample could not be quantified because of the scarcity of the purification yields. MALDIMS of the purified products of the wild-type *fucA1* gene and of Ssα-fuc are shown in (**B**) and (**C**), respectively. Peptide A and B are indicated. (**D**) LCMSMS analysis of peptide B. (**E**) The proposed frameshifting sites in the *fucA1* gene. The open and the closed arrows indicate the shifting sites A and B, respectively (for details see text). The sequence of the peptides A and B are also indicated. (**F**) Western blot of *E.coli* cellular extracts expressing the wild-type *fucA1* gene. The blot was probed with anti-GST antibodies. The pre-stained molecular weight markers were β-galactosidase (175 000), paramyosin (83 000), glutamic dehydrogenase (62 000), aldolase (47 500) and triosephosphate isomerase (32 500).

ORF SSO11867 solely (27 and 9.6 kDa, respectively). This polypeptide originated from the translational termination of the ribosome at the OCH codon of the *fucA1* N-terminal ORF (Figure 1A). The calculated ratio of frameshifting to the termination products was 5%.

Preparation and characterization of Ssα-fuc^B

To test if the full-length α-fucosidase produced by the −1 frameshifting event in site B (Ssα-fuc^B), resulting from the single P-site slippage has different properties from Ssα-fuc, whose sequence arises from the simultaneous P- and A- site slippage, we prepared the enzyme by site-directed mutagenesis. The slippery sequence in *fucA1* A-AAA-AAT was mutated in A-AAG-AAG-T where mutations are underlined. The new mutant gene was named *fucA1*^{B}. The first G, producing the conservative mutation AAA→AAG, was made to disrupt the slippery sequence and hence reducing the shifting efficiency. The second G was inserted to produce the frameshifting that results in the amino acid sequence of Peptide B. Therefore, the sequence of the two full-length mutant genes *fucA1*^{A} and *fucA1*^{B} differs only in the region of the slippery sequence: A-AAG-AAT-**TTC-GGC** and A-AAG-AAG-**TTC-GGC**, respectively (the mutations are underlined, the nucleotides in boldface were originally in the −1 frame) (Table 1).

Table 1. Nomenclature and characteristics of the α-fucosidase genes

Gene name	Status	Name of the recombinant protein	Slippery heptamer[a]
fucA1 wild type	−1 frameshifted	—	A-AAA-AAT
fucA1^A mutant	Full-length	Ssα-fuc	A-AAG-AA**T**
fucA1^B mutant	Full-length	Ssα-fuc^B	A-AAG-AA**G**
fucA1^{sm} mutant	−1 frameshifted	—	A-AAG-AAT
fucA1^{tm} mutant	−1 frameshifted	—	C-AAG-AAC

[a]Nucleotides modified by substitution and insertion mutations are underlined and in boldface, respectively.

The recombinant Ssα-fuc^B was purified up to ∼95% (Materials and Methods). Gel filtration chromatography demonstrated that in native conditions Ssα-fuc^B had the same nonameric structure of Ssα-fuc with an identical molecular weight of 508 kDa (data not shown). In addition, Ssα-fuc^B had the same high substrate selectivity of Ssα-fuc. The two enzymes have high affinity for 4-nitrophenyl-α-L-fucoside (4NP-Fuc) substrate at 65°C; the K_M is identical within the experimental error (0.0287 ± 0.005 mM) while the k_{cat} of Ssα-fuc^B (137 ± 5.7 s^{-1}) is ∼48% of that of Ssα-fuc (287 ± 11 s^{-1}). In addition, 4-nitrophenyl-α-L-arabinoside, -rhamnoside, 4-nitrophenyl-α-D-glucoside, -xyloside, -galactoside and -mannoside were not substrates of Ssα-fuc^B as shown previously for Ssα-fuc (24). This suggests that the different amino acid sequence did not

significantly affect the active site. Both enzymes showed an identical profile of specific activity versus temperature with an optimal temperature higher than 95°C (data not shown). The heat stability and the pH dependence of Ssα-fuc and Ssα-fuc^B are reported in Figure 3. At 80°C, the optimal growth temperature of *S.solfataricus*, the half-life of Ssα-fuc^B is 45 min, almost 4-fold lower than that of Ssα-fuc (Figure 3A). The two enzymes showed different behaviour at pH <6.0 at which Ssα-fuc^B is only barely active and stable (Figure 3B); however, the two enzymes showed similar values of specific activity at pHs above 6.0, which is close to the intracellular pH of *S.solfataricus (30).*

Characterization of the slippery sequence of *fucA1* in *E.coli*

The experimental data reported above indicate that the predicted slippery heptanucleotide in the region of overlap between the ORFs SSO11867 and SSO3060 of the wild-type gene *fucA1* could regulate in *cis* the frameshifting events observed in *E.coli*. To test this hypothesis, we mutated the sequence A-AAA-AAT into A-AAG-AAT and C-AAG-AAC (mutations are underlined) obtaining the *fucA1* single

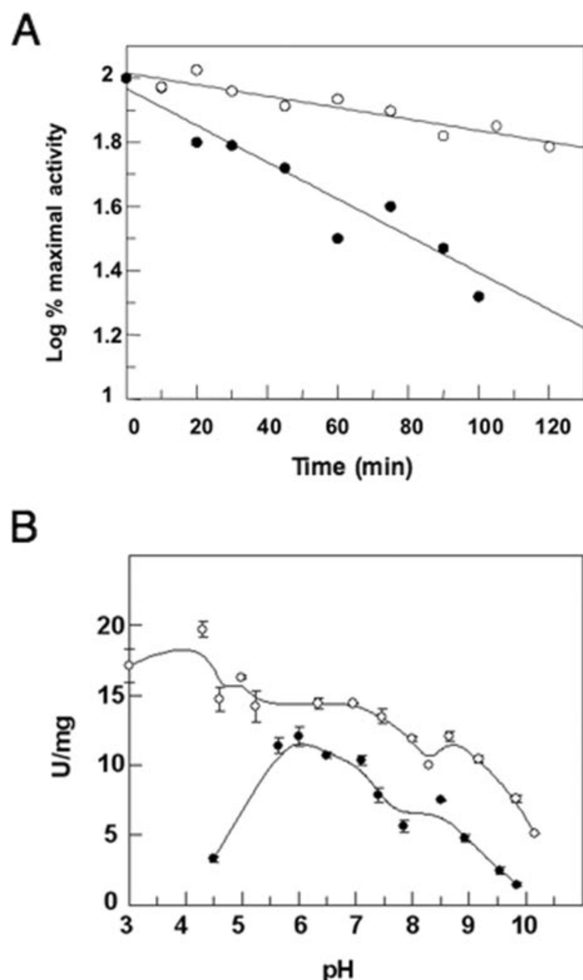

Figure 3. Comparison of the stability and pH dependence of Ssα-fuc and Ssα-fuc^B. (**A**) Thermal stability of Ssα-fuc (open circles) and Ssα-fuc^B (closed circles) at 80°C. (**B**) pH dependence of Ssα-fuc (open circles) and Ssα-fuc^B (closed circles) at 65°C.

mutant (*fucA1^{sm}*) and triple mutant (*fucA1^{tm}*) genes, respectively. It is worth noting that the mutations disrupt the slippery sequence, but they maintain the −1 frameshift between the two ORFs (Table 1).

Surprisingly, the expression of *fucA1^{sm}* in *E.coli* produced a full-length polypeptide that, after purification by affinity chromatography and removal of the GST protein, showed the same electrophoretic migration of Ssα-fuc and Ssα-fuc^B (Figure 4A). This protein was then characterized by mass spectrometry analyses following *in situ* tryptic digestion. Interestingly, the MALDI spectra revealed the presence of a single peptide encompassing the overlapping region between the two ORFs with a mass value of 1259.7 Da (peptide C; Figure 4B). The sequence of peptide C, determined from the fragmentation spectra obtained by LCMSMS analysis, was Glu-Phe-Gly-Pro-Val-Thr-Asp-Phe-Gly-Tyr-Lys (Figure 4C). Remarkably, apart from the Glu residue, this sequence is identical to that of peptide B produced from *fucA1*, indicating that in the mutant gene *fucA1^{sm}* only one of the two frameshifting events observed in the wild-type *fucA1* gene had occurred. The presence of a Glu instead of Lys was not unexpected. The mutation A-AAA-AAT→A-AAG-AAT in *fucA1^{sm}* was conservative in the zero frame of the ORF SSO11867 (AAA→AAG, both encoding Lys), but it produced the mutation AAA→GAA (Lys→Glu) in the −1 frame of the ORF SSO3060.

It is worth noting that the frameshifting efficiency of the gene *fucA1^{sm}*, calculated by western blot as described above, was 2-folds higher (10%) if compared to *fucA1* (5%) (Figure 4D). This indicates that the mutation cancelled the frameshifting site A and, in the same time, enhanced the frameshifting efficiency of site B.

In contrast, the triple mutant *fucA1^{tm}* produced in *E.coli* only the low molecular weight band resulting from translational termination (Figure 4D). No full-length protein could be detected in western blots probed with either anti-GST (Figure 4D) or anti-Ssα-fuc antibodies (Figure 4E). These data show that the disruption of the heptameric slippery sequence completely abolished the frameshifting in *E.coli* confirming that this sequence has a direct role in controlling the frameshifting *in vivo*.

Expression of fucA1 in *S.solfataricus*

To test whether *fucA1* is expressed in *S.solfataricus* we analysed the extracts of cells grown on yeast extract, sucrose and casaminoacids medium (YSM). Accurate assays showed that *S.solfataricus* extracts contained 3.4×10^{-4} units mg^{-1} of α-fucosidase activity. These very low amounts hampered the purification of the enzyme. The extracts of *S.solfataricus* cells grown on YSM revealed by western blot a band of a molecular mass >97 kDa and no signals were detected with the pre-immune serum confirming the specificity of the anti-Ssα-fuc antibodies (Figure 5A). The different molecular mass may result from post-translational modifications occurred in the archaeon or from the incomplete denaturation of a protein complex. In particular, the latter event is not unusual among enzymes from hyperthermophilic archaea (31,32). To test which hypotheses were appropriate, cellular extracts of *S.solfataricus* were analysed by western blot extending the incubation at 100°C to 2 h. Interestingly, this

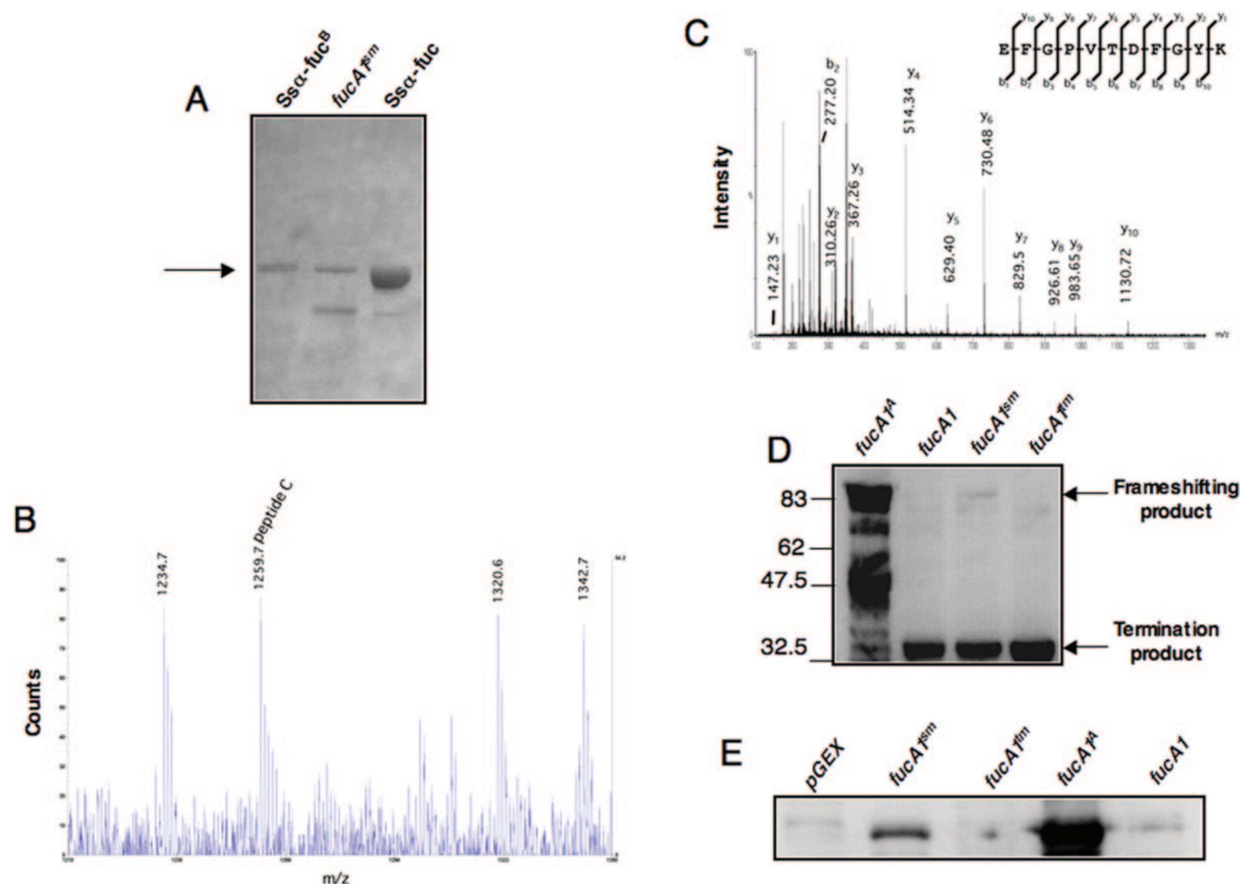

Figure 4. Analysis of the expression in *E.coli* of the mutants in the slippery sequence. (**A**) Coomassie stained 7% SDS–PAGE showing (arrow) the purified recombinant Ssα-fuc^B (1.2 μg), the product of the gene *fucA1^{sm}* (2 μg), and Ssα-fuc (4 μg). The bands with faster electrophoretic mobility result from the proteolytic cleavage of the full-length protein (25). (**B**) Partial MALDIMS spectrum of the tryptic digest from mutant *fucA1^{sm}* expressed in *E.coli*. The mass signal corresponding to peptide C encompassing the overlapping region is indicated. (**C**) LCMSMS analysis of peptide C. The amino acid sequence inferred from fragmentation spectra is reported. (**D**) Western blot of *E.coli* cellular extracts expressing *fucA1^A*, the wild-type *fucA1*, *fucA1^{sm}* and *fucA1^{tm}* genes (Materials and Methods). The blot was probed with anti-GST antibodies. (**E**) Western blot of partially purified protein samples expressed in *E.coli* fused to GST from wild-type and mutant *fucA1* genes. Cellular extracts were loaded on GST–Sepharose matrix. After washing, equal amounts of slurries (30 μl of 300 μl) were denaturated and loaded on a 8% SDS–PAGE. Extracts of *E.coli* cells expressing the parental plasmid pGEX-2TK were used as the negative control (pGEX). The blot was probed with anti-Ssα-fuc antibodies.

treatment shifted the high-molecular mass band to 67.6 ± 1.2 kDa (Figure 5B and C), which still differs from that of the recombinant Ssα-fuc, 58.9 ± 1.2 kDa, leaving the question on the origin of this difference unsolved. To try to shed some light we immunoprecipitated extracts of *S.solfataricus* with anti-Ssα-fuc antibodies and we analysed the major protein band by MALDIMS. Unfortunately, we could not observe any peptide compatible with the fucosidase because the heavy IgG chain co-migrated with the band of the expected molecular weight (data not shown).

To test if the scarce amounts of the α-fucosidase in *S.solfataricus* extracts was the result of reduced expression at transcriptional level, we performed a northern blot analysis of total RNA extracted from cells grown either on YSM or YGM media. We could not observe any signal by using probes matching the 3′ of the ORF SSO3060 (data not shown). These results suggest that *fucA1* produced a rare transcript; therefore, we analysed the level of mRNA by RT–PCR and by real-time PCR. A band corresponding to the region of overlap between the ORFs SSO11867 and SSO3060 was observed in the RNA extracted from cells grown on YSM

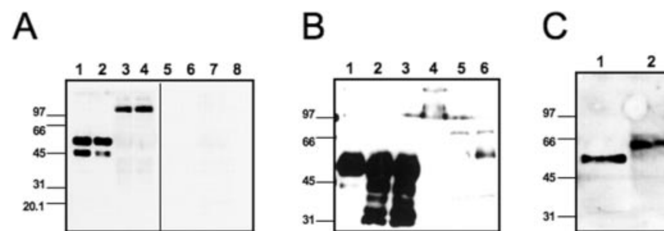

Figure 5. Analysis of the expression of the α-fucosidase in *S.solfataricus*. (**A**) Western blot analysis of recombinant Ssα-fuc (lanes 1, 2, 5 and 6, 0.14 μg) and of extracts of *S.solfataricus* cells grown on YSM (lanes 3, 4, 7 and 8, 153 μg). Samples in lanes 1, 3, 5 and 7 were not denaturated before loading. The left panel shows the blot probed with anti-Ssα-fuc antibodies; the right panel was probed with the pre-immune serum diluted 1:5 000. (**B**) Western blot analysis: recombinant Ssα-fuc (lanes 1, 2 and 3, 0.5 μg) incubated at 100°C for 5 min, 1 h and 2 h, respectively; extracts of *S.solfataricus* cells (lanes 4, 5 and 6, 1 mg) incubated at 100°C for 5 min, 1 h and 2 h, respectively. (**C**) Western blot analysis of recombinant Ssα-fuc (lane 1, 0.1 μg) incubated at 100°C for 5 min and of extracts of *S.solfataricus* cells (lane 2, 1 mg) incubated at 100°C for 2 h, respectively. The molecular weight markers were: phosphorylase b (97 000), albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000) and trypsin inhibitor (20 100).

and YGM media, demonstrating that under these conditions the two ORFs were co-transcribed (Figure 6A).

The experiments of real-time PCR shown in Figure 6B demonstrated that rRNA16S was amplified after ∼17 cycles while the amplification of *fucA1* mRNA was observed after 38 cycles, despite the fact that we used ∼2500-fold more cDNA for the amplification of *fucA1*. This indicates that the gene *fucA1* is transcribed at very low level. No significant differences in the *fucA1* mRNA level were observed in cells grown in YSM or YGM media. This is further confirmed by the analysis by western blot of the extracts of the same cells of *S.solfataricus* used to prepare the total RNAs, which revealed equal amounts of α-fucosidase in the two extracts (Figure 6C). Therefore, the low α-fucosidase activity observed under the conditions tested is the result of the poor transcription of the *fucA1* gene.

Analysis of the expression of *fucA1* in *S.solfataricus* by *in vitro* translation

To determine whether, and with what efficiency, the −1 frameshifting could be performed by *S.solfataricus* ribosomes, mRNAs obtained by *in vitro* transcription of the cloned wild-type *fucA1* gene and the mutants thereof were used to program an *in vitro* translation system prepared as described by Condò *et al*. (28). To this aim, a promoter of T7 polymerase was inserted ahead of the gene of interest to obtain RNA transcripts endowed with the short 5′-untranslated region of 9 nt observed for the natural *fucA1* mRNA (24). Autoradiography of an SDS–PAGE of the translation products (Figure 7) revealed that the wild-type *fucA1* transcript produced a tiny but clear band whose molecular weight corresponded to that of the full-length Ssα-fuc obtained by site-directed mutagenesis (24); the latter was translated quite efficiently in the cell-free system in spite of being encoded by a quasi-leaderless mRNA. Judging from the relative intensity of the signals given by the translation products of the wild-type *fucA1* and the full-length mutant $fucA1^{A}$, the efficiency of the −1 frameshifting in the homologous system was ∼10%. No signals corresponding to the polypeptides expected from the separated ORFs SSO11867 and SSO3060 (9.6 and 46.5 kDa, respectively) were observed. However, it should be noted that the product of SSO11867, even if synthesized, is too small to be detected in the gel system employed for this experiment. The larger product of ORF SSO3060, on the other hand, is certainly absent. These data unequivocally demonstrate that the ribosomes of *S.solfataricus* can decode the split *fucA1* gene by programmed −1 frameshifting with considerable efficiency producing a full-length polypeptide from the two ORFs SSO11867 and SSO3060.

Remarkably, under the same conditions at which *fucA1* drives the expression of the full-length protein, we could not observe any product from the $fucA1^{sm}$ and $fucA1^{tm}$ constructs. These data demonstrate that the integrity of the heptanucleotide is essential for the expression of the *fucA1* gene in *S.solfataricus,* thus further confirming that the gene is decoded by programmed −1 frameshifting in this organism. In addition, the lack of expression of $fucA1^{sm}$ by translation *in vitro* in *S.solfataricus* contrasts with the efficient expression of this mutant in *E.coli*, indicating that the two

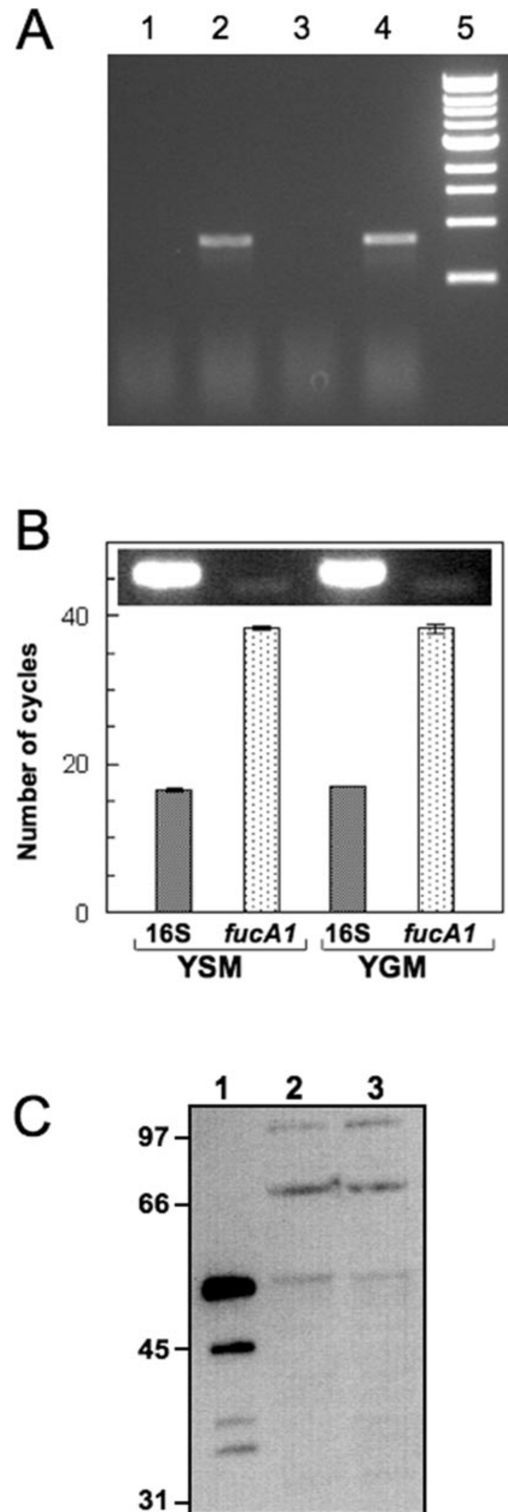

Figure 6. Analysis of the expression of *fucA1* in different media. (**A**) Agarose gel showing the products of RT–PCR encompassing the ORFs SSO11867 and SSO3060 by using total cellular RNA extracted from cells grown in YSM (lanes 1 and 2) and YGM (lanes 3 and 4); lanes 1 and 3, control (amplification of total RNA supplemented with *Taq* and without reverse transcriptase enzyme); lane 2 and 4, *fucA1*. (**B**) Comparison of the *fucA1* mRNA levels in YSM and YGM by real-time PCR. The inset shows the corresponding products found in the real-time PCR visualized by ethidium bromide staining. (**C**) Western blot of *S.solfataricus* extracts of cells grown in YSM (lane 2, 80 μg) and YGM (lane 3, 80 μg). Lane 1 recombinant Ssα-fuc (0.2 μg).

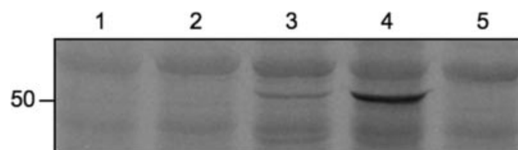

Figure 7. *In vitro* translation. Of each sample 15 µl was loaded on 12.5% acrylamide–SDS gel and the newly synthesized proteins were revealed by autoradiography. Lane 1, no mRNA added; lane 2, *fucA1*^{sm}; lane 3, wild-type *fucA1*; lane 4, full-length *fucA1*^{A}; lane 5, *fucA1*^{tm}.

organisms recognize different sequences regulating the translational frameshifting.

DISCUSSION

The identification of genes whose expression is regulated by recoding events is often serendipitous. In the framework of our studies on glycosidases from hyperthermophiles, we identified in the genome of the archaeon *S.solfataricus* a split gene encoding a putative α-fucosidase, which could be expressed through programmed −1 frameshifting (24). We tackled this issue by studying the expression of *fucA1* in *S.solfataricus* and in *E.coli* to overcome the problems connected to the scarcity of expression of the α-fucosidase gene and to the manipulation of hyperthermophiles. As already reported by others, in fact, it is a common strategy to study recoding events from different organisms in *E.coli* (23,33).

The expression in *E.coli* of the wild-type split gene *fucA1* led to the production by frameshifting of two full-length polypeptides with an efficiency of 5%. This is a value higher than that observed in other genes expressed by translational frameshifting in a heterologous system such as the proteins gpG and gpGT (0.3–3.5%) (33).

The gene *fucA1* is expressed in *S.solfataricus* at very low level under the conditions tested. In particular, the transcriptional analysis of the gene revealed that it is expressed at very low level in both YSM and YGM media. Similarly, no differences in the two media could be found by western blot probed with anti-Ssα-fuc antibodies, indicating that the low expression of the enzyme in *S.solfataricus* is the result of scarce transcription rather than suppressed translation.

Western blots allowed us to identify a specific band ~8.7 kDa heavier than that of the recombinant Ssα-fuc and experiments of translation *in vitro* showed that the wild-type gene expresses a full-length polypeptide exhibiting the same molecular mass of the recombinant protein. This demonstrates that the translational machinery of *S.solfataricus* is fully competent to perform programmed frameshifting. It seems likely that the observed discrepancy in molecular mass might arise from post-translational modifications that cannot be produced by the translation *in vitro*. Further experiments are required to characterize the α-L-fucosidase identified in *S.solfataricus*.

MALDIMS and LCMSMS analyses of the products in *E.coli* of the wild-type split gene *fucA1* demonstrated that two independent frameshifting events occurred *in vivo* in the proposed slippery site. In particular, the sequences obtained by LCMSMS demonstrate that peptide A results from a simultaneous backward slippage of both the P- and the A-site tRNAs (Figure 8A). Instead, the sequence of peptide B is the result of the re-positioning on the −1 frame of only the P-site tRNA; in fact, the next incorporated amino acid is specified by the codon in the new frame (Figure 8B). Therefore, the expression by −1 frameshifting of the wild-type gene *fucA1* in *E.coli* follows the models proposed for ribosomal frameshifting (34). We confirmed the significance of the slippery heptanucleotide in promoting the programmed frameshifting *in vivo* by mutating the putative regulatory sequence. The triple mutant *fucA1*^{tm} gave no full-length products; presumably, the mutations in both the P- and in the A-site of the slippery sequence dramatically reduced the efficiency of the −1 frameshifting as observed previously in metazoans (35). This result confirms that the intact slippery sequence in the wild-type gene *fucA1* is absolutely necessary for its expression in *E.coli*. In contrast, surprisingly, the single mutant *fucA1*^{sm} showed an even increased frequency of frameshifting (10%) if compared to the wild-type and produced only one polypeptide by shifting specifically in site B. We explained this result observing that the mutation in the P-site of the slippery sequence A-AAA-AAT→A-AA$\underline{G}$-AAT created a novel slippery sequence A-AAG identical to that controlling the expression by programmed −1 frameshifting of a transposase gene in *E.coli* (36). Therefore, apparently, the single mutation inactivated the simultaneous P- and A-site tRNA re-positioning and, in the same time, fostered the shifting efficiency of the tRNA in the P-site. It is worth noting that, instead, in *S.solfataricus*, only the simultaneous slippage is effective (Figure 8B) and even the single mutation in the slippery sequence of *fucA1*^{sm} completely annulled the expression of the gene. This indicates that this sequence is essential in the archaeon and that programmed frameshifting in *S.solfataricus* and *E.coli* exploits different mechanisms. Furthermore, since the only difference between the enzymes produced by the frameshifting sites A and B, Ssα-fuc and Ssα-fuc^{B}, respectively, is the stability at 80°C, which is the *S.solfataricus* physiological temperature, the functionality of Ssα-fuc^{B} in the archaeon appears questionable.

The reason why *fucA1* is regulated by programmed −1 frameshifting is not known. However, the physiological significance of programmed frameshifting has been assigned to a minority of the cellular genes while for most of them it is still uncertain [see (4) and reference therein; (16)]. This mechanism of recoding is exploited to set the ratio of two polypeptides such as the τ and γ subunits of the DNA polymerase III holoenzyme in *E.coli* (12). Alternatively, programmed frameshifting balances the expression of a protein, as the bacterial translational release factor 2 and the eukaryotic ornithine decarboxylase antizyme [see (4) and (18) and references therein]. In the case of *fucA1*, the polypeptide encoded by the smaller ORF SSO11867 could never be detected by western blots analyses. In addition, the modelling of Ssα-fuc on the high-resolution crystal structure of the α-L-fucosidase from *Thermotoga maritima* (25,37) showed that the *fucA1* N-terminal polypeptide is not an independent domain. Moreover, we have shown recently that SSO11867 includes essential catalytic residues (27), excluding the possibility that a functional α-fucosidase can be obtained from the ORF SSO3060 alone. Therefore, several lines of evidence allow us to exclude that

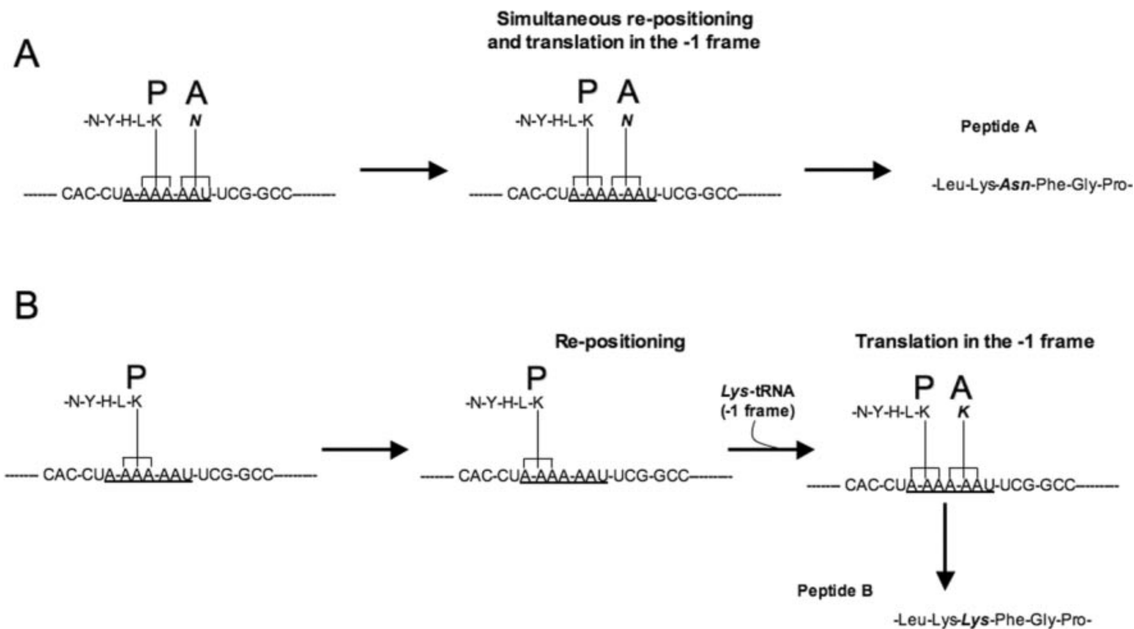

Figure 8. Proposed mechanisms of programmed −1 frameshifting of *fucA1*. (**A**) Simultaneous P- and A-site slippage; (**B**) P-site slippage. For the sake of clarity, the amino acids bound to the tRNA and in the peptides identified by LCMSMS are reported with the one- and the three-letters codes, respectively. The amino acids bound to the tRNAs shown in the A-site are highlighted in boldface and italics.

programmed −1 frameshifting is used to set the ratio of two polypeptides of the α-fucosidase from *S.solfataricus*. More probably, this translational mechanism might be required to control the expression level of *fucA1*.

Noticeably, this is the only fucosidase gene expressed by programmed −1 frameshifting. Among carbohydrate active enzymes, the only example of expression through this recoding mechanism is that reported for a gene encoding for a α(1,2)-fucosyltransferase from *Helicobacter pylori* that is interrupted by a −1 frameshifting (38). In this case, the expression by programmed frameshifting would lead to a functional enzyme synthesizing components of the surface lipopolysaccharides to evade the human immune defensive system. It is hard to parallel this model to *fucA1*. Nevertheless, the monosaccharide fucose is involved in a variety of biological functions (39). Therefore, the α-L-fucosidase might play a role in the metabolism of fucosylated oligosaccharides; experiments are currently in progress to knockout the wild-type *fucA1* gene and to insert constitutive functional mutants of this gene in *S.solfataricus*.

FucA1 is the only archaeal α-L-fucosidase gene identified so far; hence, it is probably the result of a horizontal gene transfer event in *S.solfataricus*. However, since there are no α-fucosidases genes regulated by programmed frameshifting in Bacteria and Eukarya, it is tempting to speculate that this sophisticated mechanism of translational regulation pre-existed in *S.solfataricus* and it was applied to the fucosidase gene for physiological reasons. The identification of other genes interrupted by −1 frameshifts in *S.solfataricus* would open the possibility that they are regulated by programmed −1 frameshifting. Recently, the computational analysis of prokaryotic genomes revealed that seven Archaea harbour interrupted coding sequences, but *S.solfataricus* is not included in this study (40). A computational analysis on several archaeal genomes revealed that 34 interrupted genes are present in the genome of *S.solfataricus*, 11 of these genes are composed by two ORFs separated by −1 frameshifting and could be expressed by recoding (B. Cobucci-Ponzano, M. Rossi and M. Moracci, manuscript in preparation).

We have experimentally shown here, for the first time, that programmed −1 frameshifting is present in the Archaea domain. This finding is the missing piece in the puzzle of the phylogenetic distribution of programmed frameshifting demonstrating that this mechanism is universally conserved.

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