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# TTG Serves as an Initiation Codon for the Ribosomal Protein MvaS7 from the Archaeon *Methanococcus vannielii*

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**The ribosomal protein MvaS7 from the methanogenic archaeon *Methanococcus vannielii* is a protein of 188 amino acids, i.e., it is 42 amino acids longer than previously suggested. The triplet TTG serves as a start codon. The methanogenic translation initiation region that includes the rare TTG start codon is recognized in *Escherichia coli*.**

The gene for ribosomal protein MvaS7 from the methanogenic archaeon *Methanococcus vannielii* is part of a transcriptional unit which corresponds to the eubacterial streptomycin operon. Apart from the presence of two additional open reading frames and the gene for MvaS10, the organization of the streptomycin operon of *M. vannielii* (open reading frame 1 [ORF1]-ORF2-MvaS12-MvaS7-EF2-EF1α-MvaS10) is identical to that of *Escherichia coli*. In *E. coli* the S10 gene is the first gene in the S10 operon. On the basis of the nucleotide sequence, MvaS7 was described as a protein of 146 amino acids (16). More recent homology studies of ribosomal S7 proteins from the archaea *Haloarcula marismortui*, *Halobacterium morrhuae*, and *M. vannielii* suggested that MvaS7 should be more than 40 amino acids longer than previously suggested (14).

Analysis of the nucleotide sequence preceding the ATG start codon proposed by Lechner et al. (16) revealed an amino acid sequence which shows a high degree of homology to the sequences of the other archaeal S7 proteins. Two triplets, an ATT and a TTG, which are known as unusual start codons in eubacteria (9) and archaea (18), are located in frame upstream from the originally proposed ATG start codon.

In this communication we report that MvaS7 is a protein of 188 amino acids, i.e., 42 amino acids longer than originally proposed. Furthermore, we demonstrate that TTG is used as a start codon and that the methanogenic translation initiation region containing TTG as a start codon is recognized in *E. coli*.

**Cloning and expression in *E. coli* of three size variants of the gene encoding MvaS7.** Three codons, namely, ATT (Fig. 1, position 2172), TTG (Fig. 1, position 2190), and ATG (Fig. 1, position 2317), are potential candidates for the start codon of the MvaS7 gene. To express the three possible MvaS7 variants in *E. coli* BL21(DE3) (21), we have chosen vector pET 11a (6), since the expression of cloned genes can be strictly regulated. If MvaS7, the homolog of the autoregulatory protein S7 of the streptomycin operon in *E. coli* (4), functioned in *E. coli*, we could not exclude the possibility that the basal expression level of MvaS7 in the uninduced state might affect growth of the *E. coli* host. To clone the three MvaS7 gene variants, we have used the oligonucleotides shown in Fig. 1 as primers to make PCR products containing an *Nde*I site and a *Bam*HI site. The PCR protocol and cloning of the PCR products into pET 11a via pUC 18 are detailed in reference 10. To allow cloning and efficient expression, the rare start codons ATT and TTG were replaced by an ATG. The constructs were designated pMvaS7$^{ATT}$, pMvaS7$^{TTG}$, and pMvaS7$^{ATG}$. The MvaS7 variants were specifically labelled with [$^{35}$S]methionine as follows. The T7 promoter-polymerase system (21) was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 4 μM. After 10 min, rifampin (final concentration, 300 μg/ml) was added to inhibit the *E. coli* RNA polymerase, the mixture was incubated for a further 35 min, and then the cells were pulse-labelled with L-[$^{35}$S]methionine (1,000 Ci/mmol) for 5 min. As shown in Fig. 2, all three size variants of MvaS7 are expressed in *E. coli* BL21(DE3).

**Identification of MvaS7.** Our aim was to identify MvaS7 in the two-dimensional protein pattern of the 30S ribosomal subunit of *M. vannielii*. Since we had assumed that one of the three versions of MvaS7 we had produced in *E. coli* represented the correct MvaS7, we mixed $^{35}$S-labelled MvaS7 versions with 30S ribosomal proteins and separated the proteins under the conditions described as system I in reference 17 using a Bio-Rad (Richmond, Calif.) Mini-Protean II 2-D Cell. Ribosomal subunits were prepared as detailed previously (19). Ribosomal proteins were extracted from ribosomal subunits as described by Hardy et al. (11). To obtain the $^{35}$S-labelled MvaS7 proteins, whole *E. coli* cells were subjected to an acetic acid extraction identical to that described for the ribosomal proteins (11).

A representative Coomassie blue-stained gel and an autoradiogram are shown in Fig. 3. MvaS7 was identified by superimposing the autoradiogram and the stained gel. Only the spot representing MvaS7$^{TTG}$ could be directly related to the corresponding stained protein. It was established from N-terminal sequencing of the identified 30S protein that the spot is indeed MvaS7$^{TTG}$. For this purpose the identified spot was cut out from six gels (representing about 500 ng of protein) and electrophoresed on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel to concentrate the protein. Electroblotting onto an Immobilon polyvinylidene difluoride membrane (Millipore) was performed with a buffer containing 100 mM borate (pH 9.0), 10% methanol, and 0.025% SDS at 40 V for 4 h as suggested by Jungblut et al. (13). The N-terminal sequence determination was carried out by the Wittmann Institute of Technology and Analysis of Biomolecules (Teltow, Germany). The seven amino acids (MEIKLFG) determined from the N terminus of the blotted 30S protein are identical with the amino

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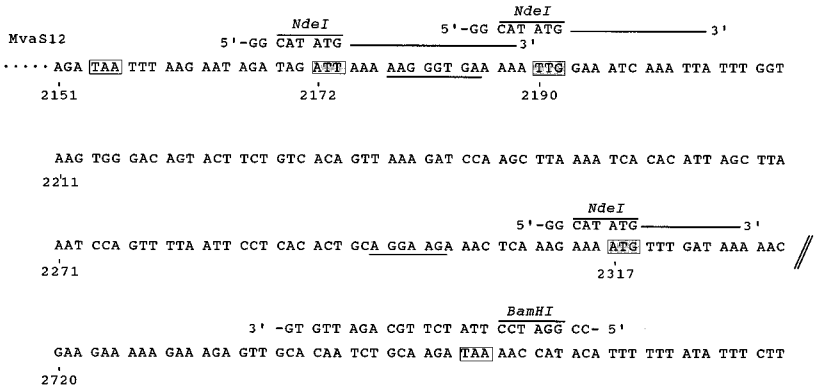


FIG. 1. Nucleotide sequences of the N- and C-terminal parts of the MvaS7 gene. The numbering of the sequence is according to the entry in the data bank (accession no. X15970). Putative start codons are given in shaded boxes. Stop codons are in unshaded boxes. Sequences that might function as Shine-Dalgarno sequences are underlined. Sense and antisense oligonucleotides used in the PCR are shown above the corresponding sequence (identical nucleotides are indicated by a bar). Double slashes represent a gap of 390 nucleotides.

acid sequence deduced from the 5′ end of the MvaS7 gene with TTG as the start codon. MvaS7, which consists of 188 amino acids, is 42 amino acids longer than previously stated (16). In bacteria, apart from the commonly used ATG, the triplets GTG, ATT, and TTG serve as initiation codons (9). In methanogens, genes initiated with TTG or, more frequently, with GTG have been identified (18). The occurrence of the rare start codon TTG in methanogenic archaea is summarized in Table 1. These genes with the rare TTG start codon do not have a common function. Furthermore, from the list of genes containing the TTG start codon one cannot conclude that the cells use TTG as a start codon to attain a substantial reduction of translation.

**The translation initiation region of the gene encoding MvaS7 functions in *E. coli*.** The 16S rRNA of methanogens has, like that from eubacteria, an anti-Shine-Dalgarno consensus sequence at its 3′ end of the 16S rRNA (12). Furthermore, upstream of virtually every methanogen open reading frame there is a sequence which shows homology to a Shine-Dalgarno sequence. The methanogen Shine-Dalgarno sequences and the other elements determining the translation initiation region, including the ATG start codon, seem to function in *E. coli* so that methanogen genes (under the control of an *E. coli* promoter) can be expressed correctly in *E. coli* (18). To see if this holds true when TTG, not ATG, is used as a start codon, we have expressed the gene for MvaS7 via its own translation initiation region in *E. coli*. To this end we have cloned the gene encoding MvaS7 plus the preceding gene encoding MvaS12 as a PCR fragment into vector pET 11a (6) and expressed them

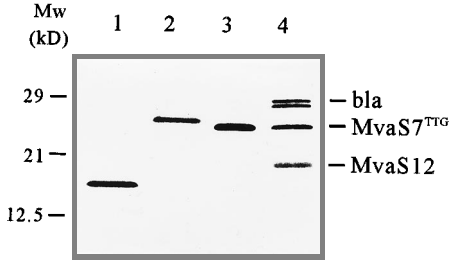


FIG. 2. Expression of the three MvaS7 variants in *E. coli* BL21(DE3) carrying pMvaS7$^{ATG}$ (lane 1), pMvaS7$^{ATT}$ (lane 2), pMvaS7$^{TTG}$ (lane 3), and pMvaS12/S7 (lane 4). The synthesis of gene products was monitored by [$^{35}$S]methionine incorporation. Cell lysates were separated in an SDS–18% polyacrylamide gel; an autoradiogram thereof is presented. Mw, molecular weight.

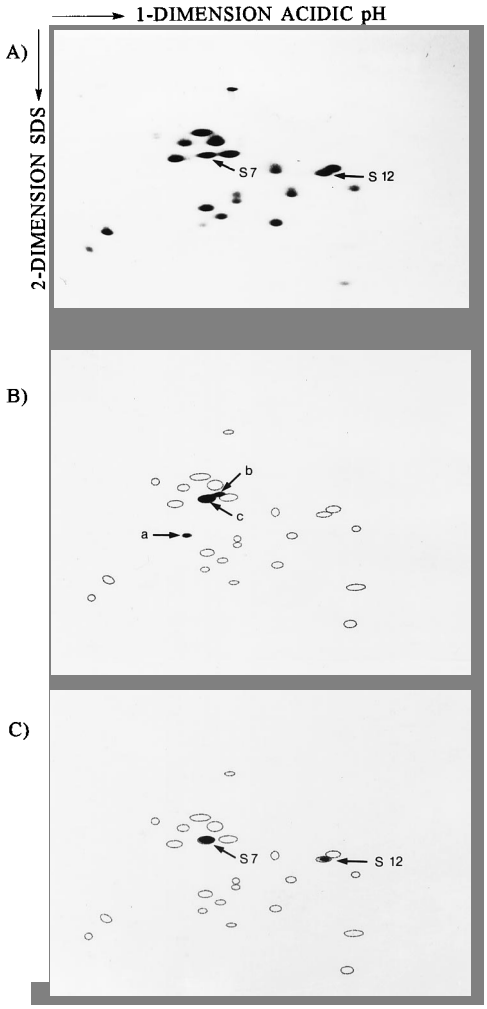


FIG. 3. Two-dimensional polyacrylamide gel electrophoresis identification of MvaS7 and MvaS12 from *M. vannielii*. (A) Coomassie blue-stained total ribosomal protein from the 30S subunit of *M. vannielii* in the gel system according to reference 17. (B) Autoradiograph of a coelectrophoresis of 30S ribosomal proteins and a mixture of $^{35}$S-labelled MvaS7$^{ATG}$ (a), MvaS7$^{ATT}$ (b), and MvaS7$^{TTG}$ (c). (C) Autoradiograph of a coelectrophoresis of 30S ribosomal proteins and $^{35}$S-labelled MvaS12 and MvaS7 produced from pMvaS12/S7. In panels B and C, the pattern of the Coomassie blue-stained ribosomal proteins is shown with dotted lines.

TABLE 1. Occurrence of the rare start codon TTG in methanogenic archaea

| Organism | Gene possessing TTG start codon | Reference or source |
|---|---|---|
| *Methanobacterium thermoautotrophicum* | *frhA* | 1 |
| *Methanobacterium thermoautotrophicum* | RNA polymerase gene Core subunit A gene | 2 |
| *Methanococcus vannielii* | Ribosomal protein S7 gene | This study |
| *Methanococcus thermolithotrophicus* | *nifH* | 20 |
| *Methanosarcina thermophila* | *pta* | 15 |
| *Methanothermus fervidus* | *pgk* | 7 |

in BL21(DE3) as described previously. In this construct, designated pMvaS12/S7, the ribosomal binding site up to the ATG start codon for the gene encoding MvaS12 is provided by the vector, whereas the gene encoding MvaS7 is translated via the TTG start codon in its native context. The expression resulted in a MvaS7 protein of the same size as that determined for MvaS7$^{TTG}$ and in a protein of about 20 kDa which represents MvaS12 (Fig. 2, lane 4). The $^{35}$S-labelled MvaS12 produced from pMvaS12/S7 was used to identify MvaS12 in the two-dimensional pattern of the 30S ribosomal protein (Fig. 3A and C). Two further plasmid-encoded proteins of about 29 and 27 kDa are synthesized from pMvaS12/S7. From their sizes, we conclude that they represent pre-β-lactamase and β-lactamase. We assume that the T7 terminator on pMvaS12/S7 was functioning inefficiently so that the β-lactamase gene was transcribed from the T7 promoter.

The MvaS7 proteins expressed from pMvaS7$^{TTG}$ and from pMvaS12/S7 are identical on two-dimensional gels (Fig. 3C); i.e., MvaS7 synthesized in *M. vannielii* and MvaS7 synthesized in *E. coli* are identical. From this we conclude that in *E. coli* the MvaS7 translation initiation region is recognized by the ribosome, as in *M. vannielii.*

A good Shine-Dalgarno sequence is situated immediately upstream of the TTG initiation codon (Fig. 1). Upstream of the originally proposed ATG start codon there is a sequence which is similar to a Shine-Dalgarno sequence, but we assume that it is not used as a ribosomal binding site as the distance to the ATG codon (13 bp) is too large. The ATT codon is not preceded by a sequence that might function as a ribosomal binding site. The translation initiation region that includes TTG as the initiation codon fulfills the criteria of a good translation initiation region in *E. coli* (8).

Alex et al. (1) reported that the *frhA* gene from *Methanobacterium thermoautotrophicum* ΔH was not expressed well in *E. coli* when the methanogen translation initiation region including the TTG initiation codon was used. We assume that the poor expression of *frhA* in *E. coli* resulted from the content of 24 AGG and AGA codons, which are the least frequently used codons in *E. coli* (22), rather than from the utilization of the rare TTG initiation codon. We have observed a similar effect. We could overexpress pMvaS7$^{TTG}$ in *E. coli* BL21(DE3) so that the gene product MvaS7 accounted for about 40% of the total bacterial protein only in the presence of pUBS 520 (3), which carries the gene for the rare tRNA$^{Arg}_{AGA/AGG}$ (5).

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