Studies on translation initiation and gene expression in *Escherichia coli*

by

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"General Conclusion" of the 1961 Cold Spring Harbor Symposia on Quantitative Biology:

If the codes in Serratia and Escherichia coli and perhaps a few other genera turn out to be the same, the microbial-chemical-geneticists will be satisfied that it is indeed universal, by virtue of the well-known axiom that anything found to be true of Escherichia coli must also be true of Elephants. (Jacques Monod and Jacob F. Cold Spring Harbor symposia on Quantitative Biology, 1961)

In the memory of my father, and all my family

Abstract

In prokaryotes, several mRNA sequences surrounding the initiation codon have been found to influence the translation process; these include the downstream region and its codon context, the Shine-Dalgarno sequence and the S1 ribosomal protein-binding site. In this thesis, the purpose has been to study the role of the downstream region and Shine-Dalgarno-like sequences on early translation elongation and gene expression in *Escherichia coli.*

The downstream region (DR) after the initiation codon (around five to seven codons), has an important role in the initiation of translation. We find that most of the codons which give very low gene expression at +2 (considering AUG as +1), reach 5 to 10 fold higher expression when those codons are positioned posteriori to +2, with the exception of the NGG codons. The NGG codons abort the translation process if located within the first five codons of the DR, due to peptidyl-tRNA drop-off. However, when the NGG codons are situated further down from the DR, the protein expression was increased at the same level of expression as in the presence of any other codon.

The Shine-Dalgarno (SD) is an important region of initiation in translation of bacteria. In spite of this, it has been found that Gram-negative bacteria could translate mRNAs with weak or non-functional SD, while the DR carries out a main role in the efficiency of translation. In addition, positions of SD and SD-like sequences are very important to direct initiation of translation in the choice between two possible initiation codons. A strong SD between two initiation sites will favor the second initiation site if it consists of a canonical start codon followed by a good DR.

The results suggest that the mRNA sequences surrounding the initiation codon: the downstream region and the Shine-Dalgarno and SD-like sequences, are very important contributors to the translation level and gene expression in *Escherichia coli*.

List of Articles

This thesis is based on the following original articles, which are referred in the text by their Roman numerals (I-III):

I. Gonzalez de Valdivia E.I, and Leif A. Isaksson, 2004. **A codon window in mRNA downstream of the initiation codon where NGG codons give strongly reduced gene expression in *Escherichia coli***. Nucleic Acids Research, **32**(17): 5198-205.

II. Gonzalez de Valdivia E.I, and Leif A. Isaksson, 2005. **Abortive translation caused by peptidyl-tRNA drop-off at NGG codons in the early coding region of mRNA**. FEBS J, **272**(20): 5306-5316.

III. Jin H., Zhao Q., Gonzalez de Valdivia E.I., Ardell David H., Stenström M. and Leif A. Isaksson, 2006. **Influences on gene expression *in vivo* by a Shine-Dalgarno sequence**. Molecular Microbiology, **60**(2): 480-492.

List of Abbreviations

aa	amino acid
ATP	adenosine triphosphate
A-site	amino-tRNA acceptor site
DB	downstream Box
DR	downstream Region
DNA	deoxyribonucleic acid
EF-G	elongation factor G
EF-Tu	elongation factor Tu
E-site	transfer RNA exit site
fMet-tRNA$_f^{Met}$	formylated initiator tRNA
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
IF- 1, 2, 3	initiation factors
IPTG	isopropyl thio-β-D-galactoside
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PTC	peptidyl transferase center
Pth	peptidyl-tRNA hydrolase
P-site	peptidyl-tRNA acceptor site
TIR	translation initiation region
Ts	temperature sensitive
tmRNA	transfer-messenger RNA
RBS	ribosome binding site
RF- 1, 2, 3	release factors
RRF	ribosome recycling factor
RNA	ribonucleic acid
rRNA	ribosome RNA
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
tRNA	transfer RNA
UTR	untranslated region

Table of Contents

1. Historical background

Nowadays, the knowledge about transcription, translation and gene expression in *Escherichia coli* is substantial. However, it has been a long journey since the first experiment made by Gregor Mendel. He established two principles of heredity now known as the law of segregation and the law of independent assortment, thereby proving the existence of paired elementary units of heredity (genes) (Dunn, 2003; Fairbanks and Rytting, 2001).

In 1928, Fred Griffith discovered that a non-pathogenic R pneumoccoci mutant could be transformed into a pathogenic S pneumoccoci form (Stryer, 1988). This result was the first step paving the way for the elucidation of the chemical nature of the transforming principle, the deoxyribonucleic acid (DNA). Sometime later, in 1944, Oswald Avery and coworkers (Avery, 1944) demonstrated that a nucleic acid of the deoxyribose type is the transforming principle of *Pneumoccocus* type III (73 years after Mendel). Supporting this finding, DA Hershey and Martha Chase confirmed the genetic role of DNA in a virus that infects the bacterium *Escherichia coli* (Hershey and Chase, 1952).

A crucial year in the development of this new era of molecular biology was 1953. The race between different groups to deduce the three-dimensional structure of DNA was set. It was James Watson and Francis Crick that published the correct molecular structure of nucleic acid (Watson and Crick, 1953). Their proposed structure immediately suggested a mechanism of replication in which one of each DNA daughter molecule is newly synthesized, where the other strand is passed on unchanged. This idea was tested and confirmed experimentally by Meselson and coworkers (Meselson and Stahl, 1958). Advancing frontier further of the nucleic acid molecular biology was the work of Arthur Kornberg, who isolated an enzyme from *Escherichia coli* that catalyzes the synthesis of DNA, DNA polymerase (Kornberg, 1960).

In 1960, a new decade started with very important discoveries about the RNA. It was found that different RNA molecules are involved in the translation and *de novo* polypeptide synthesis. Messenger RNA (mRNA) (Jacob and Monod, 1961), transfer RNA (tRNA) and ribosomal RNAs (rRNAs) are components of the protein synthesis machinery (Woese, 2001). These RNAs are synthesized by the RNA polymerase (Hurwitz, 2005). By this time, after the discovery by Palade of a ribonucleotide particle binding the endoplasmatic reticulum in eukaryotic cells (Palade, 1955; Palade and Siekevitz, 1956), a ribonucleotide particle was also found and isolated from *Escherichia coli*. These were characterized and named ribosomes (Kurland, 1960; Roberts, 1958; Tissieres, 1960).

Several groups studied the relation between the RNA and protein. It was shown that ribosomes are connected with protein synthesis (Hoagland, 1960; Hoagland, 1958; Zamecnik, 1960). Many efforts were made at that time to reveal the relation between the genetic code and the twenty amino acids, accomplished by Nirenberg and Mathei from 1961 to 1965 (Nirenberg, 2004) as well as Khorana and coworkers (Khorana *et al.*, 1966; Nishimura, 1965). Experiments done at Charles Yanofsky's lab showed the relation or colinearity of the gene with the novo polypeptide synthesis (Sarabhai *et al*., 1964; Yanofsky *et al.*, 1964).

The idea appeared that in the development and evolution of life on the earth, the RNA world appeared first and preceded the appearance of protein synthesis (Crick, 1968; Orgel, 1968; Woese, 1968). But, it was not until much later that this idea took over, when the ribozymes were discovered in 1982 (Cech, 2002) which led to the discussion of the role of RNA in the origin of life and the notion of an "RNA world".

However, DNA replaced RNA as a genetic material at some time during evolution because its double helix is a more stable and reliable store of genetic information than a single stranded RNA (Gilbert, 1986; Joyce and Orgel, 1999). RNA molecules have an extensive role in contemporary biology, especially with regard to the most fundamental highly conserved cellular processes. RNA is involved as a primer in

DNA replication. It acts as a messenger that carries genetic information to the translation machinery, and is a catalyst that lies at the heart of the ribosome (Joyce, 2002). However, recently a RNA system where no proteins seems to be required for either sensing the concentration of the metabolic end-product or switching off gene expression has been found (Cech, 2004; Winkler *et al*., 2004).

The results mentioned above were the milestones of a new era in Science, which is creating an explosive symbiosis in nature between the genetic material and the human being for the times to come. This is the real biology, the biology that addresses "the most challenging intellectual problem of all time, that is, Mankind's eternal question, how we came to be". And in the center of it all sits the problem of translation, how it works, how it arose, and how its evolution transmogrified[1] an ancient RNA world (Brenner, 1998; Woese, 2001).

2. Introduction to the translation process

The initiation codon AUG and mRNA surrounding sequences are important in gene expression. These sequences are involved in the regulation of the translation process, gene expression, and protein biosynthesis in bacteria.

The central role of this process is played by the ribosome, the organelle which translates the genetic information of an mRNA molecule into a sequence of amino acids (Marquez *et al.*, 2002). Almost 50 % of the bacterial mass is devoted to ribosomes. The ribosome, a large ribonucleoprotein consists of two subunits in all species. In bacteria, the subunits are designated 30S (the small subunit composed of 21 proteins and 16S rRNA) and 50S (the large subunit composed of 34 proteins and 2 rRNAs: 5S and 23S rRNAs), and together they make up the 70S ribosome. Each subunit has three binding sites for tRNA, designated the A-site (aminoacyl) which

[1] Transmogrification: the process or result of changing from one appearance, state, or phase to another.

accepts the incoming aminoacylated tRNA; P-site (peptidyl), which holds the tRNA with the nascent peptide chain; and E-site (exit), which holds the deacylated tRNA before it leaves the ribosome (Ramakrishnan, 2002). The movement of tRNA and mRNA through the ribosome is a complicated process that combines high speed with high accuracy (Green and Noller, 1997). Base pairing between the codon in mRNA and the anticodon in tRNA is the ultimate basis for selection of the correct tRNA for the participation in the addition of a new amino acid to the growing polypeptide chain (Ramakrishnan, 2002).

Translation is a very complicated process, where the ribosome is the central figure in the protein synthesis, and it has been stated in a landmark phrase from Noller and Woese (Noller and Woese, 1981): ...”Our approach is based on the paradigm that the mechanism of translation is defined by the RNA component of the ribosome”… Many studies and new approaches to learn how ribosomes work were made in the last decades. A clear example of this activity is the structural determination at a resolution of less than 25 Å down to almost atomic resolution (Ban *et al.*, 2000; Schluenzen *et al*., 2000; Wimberly *et al.*, 2000; Zhao *et al.*, 2004a, b).

These important breakthroughs of understanding ribosome structures will prompt for new interesting discussions about the molecular mechanism of translation and gene expression in bacteria. In this study, we intend to provide some contribution to the general knowledge of the early coding region and its influence on gene expression, and hopefully, to the general comprehension of the translation mechanism in the model bacterium *Escherichia coli*.

3. The translational process

Protein biosynthesis includes mRNA, various protein factors, tRNAs, many proteins and the ribosome. The enzymatic reactions are carried out in the polypeptide

synthesis with the help of ATP and GTP hydrolysis that supply the necessary energy to drive the process at a high speed and accuracy (Ninio, 1975). The translational process consists of four phases: Initiation, elongation, termination and recycling.

3.1. The Initiation phase

The main elements of a canonical translation initiation site include the initiation triplets, AUG (83%), GUG (14%) and UUG (3%), used for initiation of bacterial genes (Blattner *et al*., 1997). In addition, the purine-rich Shine-Dalgarno (SD) sequence is complementary to the 3' end region of the 16S rRNA (Shine and Dalgarno, 1974). A spacer of variable length separates the SD sequence from the initiation triplet (Gualerzi and Pon, 1990; McCarthy and Brimacombe, 1994; Ninio, 1975). The fMet-$tRNA_f$ is used for initiation in bacteria. It has been characterized and it was noted that it differs in structure from the other tRNAs (Gillum *et al*., 1975; Roe *et al*., 1975). The process involves three initiation factors IF1, IF2 and IF3 (Gualerzi and Pon, 1990).

The IF3 is known to bind strongly to the 30S subunit and to prevent its association with the 50S subunit. It also helps in the selection of initiator tRNA ($tRNA^{fMet}$) by destabilizing the binding of other tRNAs in the P-site of the ribosome (Hartz *et al.*, 1990). IF3 can promote the binding of a small hairpin that mimics the tRNA ($tRNA^{fMet}$) anticodon stem and loop in competition with other tRNAs (Hartz *et al.*, 1989). IF3 influences the kinetics and fidelity of codon-anticodon recognition of the fMet-tRNA (Meinnel *et al.*, 1999; O'Connor *et al*., 2001).

The IF2 increases the specificity of the initiation complex for $tRNA^{fMet}$ as a response to the initiation codon, but only if fMet-$tRNA^{fMet}$ is used (Hartz *et al.*, 1989). No direct location of IF2 has been determined, but since it is known to bind the aminoacyl end of initiator tRNA in the P-site, as well as to interact with IF1, a

model could be proposed in which it binds together with IF1 in the A-site (Roll-Mecak *et al.*, 2000). However, models in which IF2 and IF1 in concert mimic the anticodon loop, anticodon stem, D-loop, and D-stem of A-site bound tRNA, are not relevant in the light of the new structural knowledge and recent biochemical data (Laursen *et al.*, 2005).

A role for IF1 becomes apparent only when 70S ribosomes are used instead of 30S subunits to form the pre-initiation complex. The location of IF1 in the ribosomal A-site suggests that by blocking the premature access of aminoacyl-tRNAs to this site the factor may play an "initiation fidelity function" (Boelens and Gualerzi, 2002). However, despite of many years of work, the order in which the factors bind and are released *in vivo*, and what they do with the conformation of the ribosome, have not been definitively elucidated (Laursen *et al.*, 2005; Ramakrishnan, 2002).

3.2. The Elongation phase

The elongation phase starts with a peptidyl-tRNA occupying the ribosomal P-site with an empty A-site. The A-site is then filled with an aminoacyl-tRNA (aa-tRNA) that has a complementary anti-codon to the codon in the mRNA according to the Watson model of a two site mechanism (Watson and Crick, 1953). The aminoacylated tRNA is brought into the A-site as a ternary complex with EF-Tu and GTP.

The structural recognition of codon-anticodon base pairing is only one feature of the decoding process. The selection of tRNA begins with the binding of the EF-Tu ternary complex after its activation with a GTP molecule, which presents the aminoacylated tRNA to the decoding site at an angle that will allow the proofreading step (Stark, 1997). The EF-Tu•GTP•aa-tRNA (ternary complex) is stabilized in the A-site by the codon/anticodon interaction of the tRNA with the mRNA and, possibly, the ribosome. Thus the codon-anticodon interaction generates an activation signal

that is transmitted to the G domain of EF-Tu and leads to the formation of the activated GTPase state of the ribosome•EF-Tu•aa-tRNA•GTP complex that is followed by GTP hydrolysis.

Hence, the conformation of EF-Tu switches from a GTP to a GDP form, which has a greatly reduced affinity for aa-tRNA and the ribosome. Therefore, the aa-tRNA is released from EF-Tu•GDP, accommodates in the A-site and takes part in the peptidyltransferase reaction, while EF-Tu•GDP dissociates from the ribosome (Rodnina, 2000). EF-Ts binds to the EF-Tu•GDP to release the GDP molecule. Since the concentration of GTP in the cytoplasm is much higher than the concentration of GDP, GTP will preferably bind to an empty nucleotide site in EF-Tu (Liljas, 2004).

As a result, this leads to the release of the aminoacyl end of A-site tRNAs from EF-Tu and swinging of the CCA-end into the peptidyl transfer center (PTC) of the 50S subunit in a process called accommodation (Valle *et al*., 2003). The phase is set for the formation of a peptide bond, which involves the transfer of the peptide chain from the P-site tRNA to the amino acid of the A-site tRNA. This reaction is catalyzed by peptidyl transferase, an enzyme activity of the 50S subunit. When the aminoacyl end of A-site tRNA enters the peptidyl transferase center, peptide bond formation occurs rapidly and spontaneously (Pape *et al*., 1998).

After the discovery of catalytic RNA (Kruger *et al.*, 1982; Zaug *et al.*, 1983) and biochemical evidence for the role of 23S RNA in peptidyl tranferase (Green and Noller, 1997) the notion has been accepted that ribosomal RNA catalyzes peptidyl transfer. Following peptidyl transfer, the ribosome has a deacylated tRNA in the P-site and peptidyl tRNA in the A-site. Translocation of the tRNAs and mRNA to the next codon is facilitated by EF-G, which also is a GTPase.

A third tRNA binding site on *Escherichia coli* ribosomes, the E-site (exit) is specific for the deacylated tRNA molecule. The E-site was discovered and accepted as a final site for the tRNA before it dissociates from the ribosome (Grajevskaja *et al.*, 1982; Kirillov *et al*., 1983; Lill *et al*., 1984; Rheinberger *et al.*, 1981;

Rheinberger, 1980). In contrast to earlier proposals, it is clear that the E-site tRNA interacts with both ribosome subunits (Yusupov *et al.*, 2001). Due to the tRNA transfer to the E-site, the ribosome is ready for decoding the next codon during elongation. It has deacylated tRNA in the E-site, peptidyl tRNA in the P-site, and an empty A-site that is ready to receive the next cognate ternary complex (Ramakrishnan, 2002).

3.3. Termination and Recycling phases

In bacteria, three release factors: RF1, RF2 and RF3, have been found. RF1 responds to UAG and UAA and RF2 responds to UAA and UGA stop codons (Kisselev *et al*., 2003; Scolnick, 1968). The action of one or another of these protein factors leads to the hydrolysis of the ester linkage between tRNA and the polypeptide on the ribosome and gives release of the completed polypeptide (Ehrenberg *et al.*, 2000). RF3 was discovered to catalyze the removal of release factors RF1 or RF2 from the ribosome, but RF3 itself lacks codon specificity (Freistroffer *et al.*, 1997; Goldstein *et al*., 1970; Milman *et al*., 1969).

The RF3 possesses GTPase activity (Freistroffer *et al*., 1997; Pel, 1998). Thus, part of the RF3 function is to catalyze recycling of RF1 or RF2 and maintain adequate levels of free factors (Kisselev and Buckingham, 2000). The other aspect of RF3 function is to accelerate the transition from termination to ribosome recycling, the post-termination state. This process requires the binding of still another factor, ribosome recycling factor (RRF) to the ribosome at A-site overlapping that of RF1 or RF2 (Pavlov *et al.*, 1997).

The RRF along with EF-G is required for the recycling process, where the components that are bound to the mRNA are recycled (Janosi *et al*., 1996). The recycling process will prevent a random reinitiation of protein synthesis (Janosi *et al.*,

1998). A third protein, the initiation factor IF3, was isolated as a factor involved in splitting the 70S ribosome into subunits (Karimi *et al*., 1999). However, it has recently been shown that hydrolysis of GTP in the ribosome-RRF•EF-G complex leads to a dissociation of the intact 70S ribosome from the mRNA and tRNA (Kaji *et al.*, 2001).

4. Translation and gene expression

4.1 mRNAs translation initiation regions

In *Escherichia coli*, mRNAs contain different sequences that influence the efficiency of translation initiation. The prokaryotic translational initiation region (TIR) includes the region commonly referred to as the ribosome-binding site (RBS) as well as the bases extending beyond the 5' and 3' limits of the RBS (Gualerzi *et al.*, 2000).

The minimal definition of the RBS comprises the Shine-Dalgarno region and the start codon plus the bases in between (McCarthy and Gualerzi, 1990) (Fig. 1). The downstream region (DR) after the canonical initiation codon (AUG, GUG or UUG) is a narrow window comprising five to seven codons having a strong influence in translation and gene expression (Gonzalez de Valdivia and Isaksson, 2004; Looman *et al*., 1987; Stenström *et al.*, 2001a).

Studies underline the view that initiation codon sequences and mRNAs secondary structure(s) as well as the Shine-Dalgarno (SD) sequence and its complementary binding to the anti-SD (ASD) in 16S rRNA, are major determinants for the translation initiation efficiency of prokaryotic mRNAs (Draper, 1996; Gold, 1988). However, other studies have revealed that the SD sequence is not strictly essential for translation (Calogero *et al*., 1988; Tedin *et al.*, 1999).

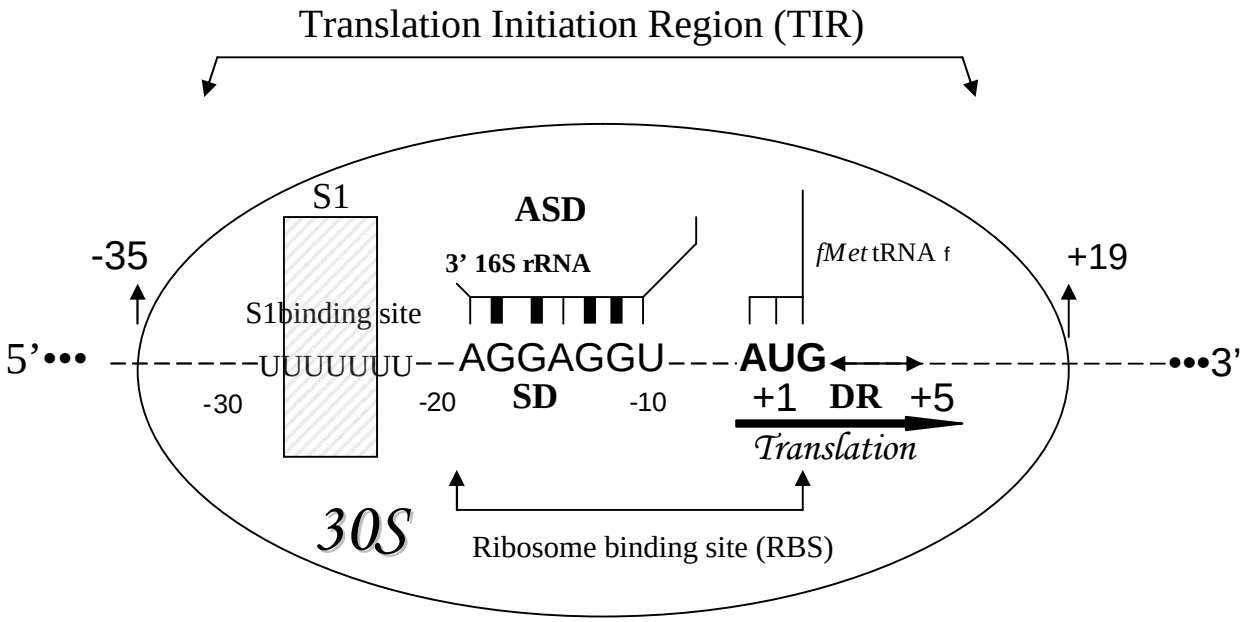

Fig. 1. Schematic description of the eubacterial translation initiation region (TIR). The figure is a compilation of data from different authors (Boni *et al.*, 1991; Gonzalez de Valdivia and Isaksson, 2004; Hüttenhofer and Noller, 1994; McCarthy and Gualerzi, 1990; McCarthy and Brimacombe, 1994; Stenström *et al.*, 2001a; Stenström *et al.*, 2001b).

Also other sequences surrounding the initiation codon contribute to the efficiency of the initiation signal (McCarthy and Brimacombe, 1994). According to an alternative model, translational enhancers within the mRNA 5' untranslated regions (5' UTR) serve as targets for the key mRNA-binding ribosomal protein S1 (Boni *et al.*, 1991; Zhang and Deutscher, 1992). This enhancer region is essential for the translation machinery of Gram-negative organisms (Komarova *et al*., 2002).

4.2. Downstream region following the AUG translation initiator codon

Several translational enhancer elements besides the upstream SD sequence and the start codon itself have been reported to stimulate translation of various prokaryotic and eukaryotic reporter mRNAs in bacteria (McCarthy and Brimacombe, 1994; Shine

and Dalgarno, 1974). In addition to the elements mentioned above, a translational enhancer downstream of the initiation codon has been described as a translational *cis*-acting element which influences the translation of leadered mRNA, thereby mediating independent and efficient initiation of translation (Gualerzi *et al.*, 2000). This has been called the downstream box (DB) or downstream region (DR) (Stenström *et al*., 2001a; Stenström *et al*., 2001b).

The DB was originally described as a translational enhancer element of approximately 8-13 nucleotides that is complementary to nucleotides 1469-1483 in the helix 44 of the 16S rRNA (antidownstream box-ADB) and located downstream of the initiation codon in *E.coli* and bacteriophage mRNAs (Etchegaray and Inouye, 1999; Faxén *et al.*, 1991; Shean and Gottesman, 1992; Sprengart *et al.*, 1990). However, Resch and coworkers concluded that DB elements do not influence translation of leaderless mRNA (Resch *et al.*, 1996). In agreement with previous data, it has also been shown that ribosomes carrying an inversion in the anti-DB region of a 16S rRNA translate leadered mRNA with or without DB after the initiation codon with the same efficiency as wild type ribosomes (Moll *et al*., 2001; O'Connor *et al*., 1999).

4.3. Codon composition downstream of the AUG start codon

Studies in *Escherichia coli* showed that open reading frames (ORFs) of highly expressed proteins show a strong avoidance of low usage codons, because such low usage codons may be more difficult to translate (Grosjean and Fiers, 1982; Konigsberg and Godson, 1983).

The presence of low usage codons or rare codons is avoided in nature due to a natural selection for efficiently translated codons (Cancilla *et al.*, 1995; Faxén *et al.*, 1991; Kurland, 1987; Sharp and Li, 1986, 1987). Particularly, studies on CUA and

AGG codons have shown that it must be translated slowly even if the number of codons in the mRNAs of the cell is low (Bonekamp *et al.*, 1989; Curran and Yarus, 1989; Varenne *et al*., 1989). Some results point out a correlation between protein synthesis machinery and *in vivo* protein folding, and their relation with the presence of slow regions on mRNA. However, some rare codons, such as CUA and AGG, which are translated very slowly, are avoided in slow regions, contrary to expectation that rare and slowly codons should be overrepresented in the slow regions (Thanaraj and Argos, 1996). The authors suggest the avoidance of these codons in the slow regions because they may induce dissociation of the translation complex and cause a deleterious rather an intentional pause.

Other minor codons such as AGA, CUA, UCA, AGU, ACA, GGA, CCC, and AUA are used preferentially within the first 25 codons in *E.coli* genes (Chen and Inouye, 1990). The authors suggest that such preference for the minor codons in an early gene section may modulate gene expression by premature termination of translation, thereby avoiding unnecessary translation of a large part of the mRNA.

Most of these codons decrease gene expression when they are situated at the +2 position downstream of the AUG initiation codon (Looman *et al*., 1987; Stenström *et al.*, 2001a).

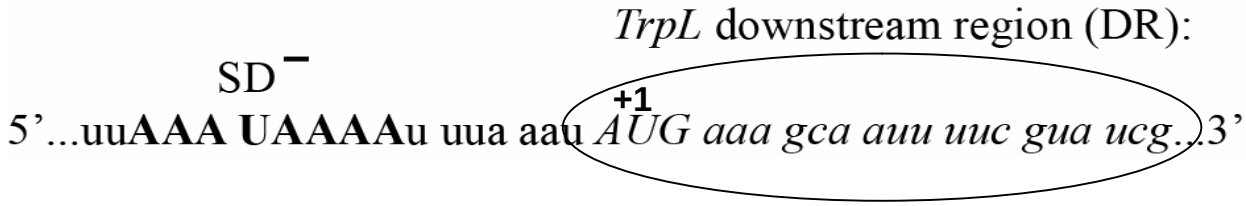

Fig. 2. *TrpL* sequence with a non functional Shine-Dalgarno (SD), a Downstream Region (DR) after a canonical start codon (AUG). Codons provoking low, medium and high level of translation and gene expression were subcloned within the DR.

Several investigations have proposed that the maximum gene expression is dependent on the downstream context of codons and therefore, the DR following the initiation codon could act independently of the nature of the initiation codon on the efficiency of translation initiation. Hence, the data suggest that the levels of gene

expression is influenced by the early codon sequence in the mRNA (Gonzalez de Valdivia and Isaksson, 2004; Stenström and Isaksson, 2002; Varenne *et al.*, 1989).

The presence of these rare codons with limited availability of the decoding aminoacyl-tRNA, would raise translational difficulties in their decoding, and the ribosome would stall while waiting for the appropriate tRNA. A stalled ribosome is more likely to produce alternative paths of translation at hungry codons cognate to the limiting species (Del Tito *et al.*, 1995; Gallant and Lindsley, 1998; Gustafsson *et al.*, 2004). The translational errors produced due to a stalled ribosome would include binding of non-cognate aminoacyl-tRNA species, frameshifting, tmRNA action and peptidyl-tRNA release (Del Tito *et al*., 1995; Gallant and Lindsley, 1998; Ivanov *et al.*, 2002; Menninger, 1976; Withey and Friedman, 2002).

Inhibition of gene expression by consecutive rare and low usage codons has been analyzed (Ohno *et al.*, 2001; Zhang *et al*., 1994). These codons have earlier been tested *in vivo* by using gene expression constructs containing a cluster of two to five of these low usage codons (Chen and Inouye, 1990, 1994; Gao *et al*., 1997; Goldman *et al.*, 1995; Olivares-Trejo *et al.*, 2003; Rosenberg *et al*., 1993; Spanjaard and van Duin, 1988; Zahn and Landy, 1996). The results obtained from these studies revealed that the presence of consecutive low usage codons at the very beginning of the gene produce an abortive translation. This abortive translation or ribosomal release without stop codon could be due to a drop-off effect.

4.4. Drop-off, a processivity error

All errors which prevent the completion of a full length protein are referred to as processivity errors (Jørgensen and Kurland, 1990). During translation the peptidyl-tRNA may drop-off from the working surface of the ribosome-mRNA complex (Kurland, 1992). The proteolytic destruction of the incomplete nascent polypeptide is

initiated by separation of the tRNA from the polypeptide by peptidyl-tRNA hydrolase (Pth) (Atherly and Menninger, 1972; Garcia-Villegas *et al.*, 1991; Heurgue-Hamard *et al*., 1996; Menninger, 1976).

From the accumulation rate of peptidyl-tRNA in a temperature sensitive peptidyl-tRNA hydrolase mutant *pth*(Ts), it has been estimated that the spontaneous ribosomal peptidyl-tRNA dissociation rate is one per 2600 to 4000 translated codons (Jørgensen and Kurland, 1990; Menninger, 1976).

Drop-off is probably the dominant part of the overall processivity error in the cell (Kurland, 1992). The dissociation of peptidyl-tRNA from ribosomes has been associated with translation. Two mechanisms have been described for drop-off. One is the "editing mechanism" proposed by Menninger (Menninger, 1977) and the second is processivity error in translation, suggested by other researchers (Dong and Kurland, 1995; Jørgensen and Kurland, 1990; Kurland *et al*., 1996). Recently, it has been suggested that the drop-off process is catalyzed by translation initiation factors (Karimi *et al*., 1998) and termination factors (RRF, EF-G and RF3) (Heurgue-Hamard *et al.*, 1998). The spontaneous drop-off rates of Na-Phe-Phe-tRNA^{Phe} from the A/P as well as the P/P state have been measured (Karimi and Ehrenberg, 1996). They have also showed that peptidyl-tRNA is more stably bound to the ribosome in the P/P than in the A/P hybrid state.

In most textbooks, the only described function of the initiator factors is to ensure a correct initiation of translation. Recent studies have suggested that IF1 and IF2 could stimulate drop-off of short mini-peptides, the effect decreasing with increasing length of the nascent polypeptide. In addition, drop-off rates will be different for different peptidyl-tRNA species during the translation process, and the context of the codon in the A-site may be important for the drop-off rate (Dinçbas *et al*., 1999).

The tRNA pools in bacterial cells and their importance for *de novo* synthesis have been studied for many years. Starvation of tRNAs could lead to inhibition of protein synthesis. This can occur by the drop-off process, as has been found to give the

depletion of $tRNA^{Lys}$ from the tRNA pool by its sequestration in cells deficient in peptidyl-tRNA hydrolase (Heurgue-Hamard *et al.*, 1996).

Codon context and rare codons have been associated with a low level of translation in gene expression. New development in this area shows that drop–off in the early coding region could be the phenomenon behind low gene expression in the presence of a pair of rare codons in natural genes (Olivares-Trejo *et al.*, 2003), and single codons in minigenes (Cruz-Vera *et al.*, 2003). The drop-off provoked by single NGG codons within the downstream region after the AUG initiator codon in an open reading frame is reported in the present work (Gonzalez de Valdivia and Isaksson, 2005).

4.5. Shine-Dalgarno sequence

The prokaryotic messenger RNA is apparently recognized by the ribosome through a sequence named Shine-Dalgarno (SD). Although this sequence is abundantly present in mRNA in bacteria, it is absent in eukaryotes.

The SD is a conserved, purine rich region described by Shine and Dalgarno (Shine and Dalgarno, 1974) and can be localized within the TIR (Schneider *et al.*, 1986). It is located upstream of the initiation codon and is complementary to a sequence close to the 3' terminus of the 16S rRNA. The interaction between the mRNA SD sequence and anti-SD sequence of 16S rRNA has been elegantly confirmed by showing that site-directed mutagenesis of the anti-SD sequence (1535-1540) in plasmid-encoded rRNA genes affects the levels of translational efficiency of mRNA by complementary base pairing effects (Hui and de Boer, 1987; Jacob *et al.*, 1987). Data from x-ray crystallographic studies shows that the interaction between the SD and the anti-SD sequences is located at a large cleft between the head and the back of the platform on the 30S ribosomal subunit (Ogle *et al.*, 2001; Yusupova *et al*., 2001)

The ribosome does not need a perfect distance between the SD and the AUG initiator codon for the initiation of translation. In spite of this, when the SD resides within four nucleotides from the AUG, or when it is located as far as 13 nucleotides from the AUG, gene expression is decreased drastically (Chen *et al.*, 1994; Kozak, 1999; Ringquist *et al*., 1992). However, many authors have shown that ribosomes from Gram-negative bacteria, e.g. *Escherichia coli* are able to translate mRNAs from a variety of sources in a manner independent of the strength of the SD regions (Roberts and Rabinowitz, 1989). It is suggested that the function of SD sequences is to ensure a high concentration of the initiation triplet near the ribosomal peptidyl-tRNA binding site (Calogero *et al.*, 1988).

Other authors considered that the function of the SD sequence is to anchor the mRNA on the ribosome transiently to allow the kinetic selection of a potential initiation triplet whose local concentration is increased in the proximity of the P decoding site (Gualerzi and Pon, 1990). Olsthoorn and co-workers suggest another function for the SD sequences: a co-evolution of RNA helix stability and Shine-Dalgarno complementarity in a translational start region. These findings support a previously published model in which the SD interaction helps the ribosome to melt the structure in a translation-initiation region (Olsthoorn *et al.*, 1995).

4.6. mRNA binding site for ribosomal protein S1.

In addition to the SD sequence, other mRNA *cis* elements have been found to have a considerable positive effect on the translation efficiency, due to their possible involvement in the recruitment of ribosomes in the first step of translation initiation (Gualerzi *et al.*, 2000; McCarthy and Gualerzi, 1990; McCarthy and Brimacombe, 1994). One such region is the translational enhancer within the mRNA 5' untranslated regions (5'UTR) which is regarded as responsible for the SD-

independent pathways of initiation complex formation on SD-less mRNA (Komarova *et al*., 2002).

This translation enhancer within the mRNA 5' UTR serves as a target for a key mRNA-binding ribosomal protein S1 (Boni *et al.*, 1991; Zhang and Deutscher, 1992). Protein S1 is located at the junction of the head, platform, and main body of the 30S subunit (Sengupta *et al.*, 2001). Evidence for a direct interaction of S1 with 11 nucleotides of the mRNA, immediately upstream of the Shine-Dalgarno sequence, explains the protein's role in the recognition of the 5' region of mRNA (Sengupta *et al.*, 2001). Protein S1 is also essential for translation of highly structured mRNAs (Szer *et al*., 1975; Van Dieijen *et al*., 1975).

S1 has been suggested to assist in the positioning of the 30S subunit in close proximity to the translational start site by destabilizing secondary structures (de Smit and van Duin, 1994a) as well as in the formation of the translation initiation complex at an internal ribosome binding site. However, S1 is not required for in vitro 30S initiation complex formation on leaderless mRNAs (Moll *et al.*, 2002; Tedin *et al.*, 1997). Ribosomal S1 and IF3 seems to be indispensable for translation initiation complexes and their stability with leadered mRNA (Boni *et al*., 1991). In contrast, in the case of leaderless mRNA, S1 apparently contributes to IF3-dependent destabilization of translation initiation complexes formed at 5'-terminal start codons (Moll *et al.*, 1998; Moll *et al.*, 2002).

5. The aim of this study

Escherichia coli continues to be a useful host for gene expression of proteins with relevance for the biomedicine sector. Although the molecular biology of *Escherichia coli* is rather basic and is economically viable for biotech exploitation, the study of

the early translation process is very important for a better understanding of the translation mechanism and early gene expression.

The level of gene expression in bacteria is the result of several factors such as the intracellular mRNA concentration and stability, secondary structure, tRNA pool, codon context and the efficiency of its translation by the ribosome translation complex. Previous results cited elsewhere in this thesis, have shown the importance of all those factors on translation efficiency and gene expression. Our work has been focused on the downstream and upstream regions of the initiation codon and their influences on gene expression. From this point of view, the aim of the work presented in this thesis has been analyzed as follows:

- Codon effect within the downstream region, following the canonical initiation codon, and its influences on gene expression in *Escherichia coli*.
- Low gene expression by the four particular early codons: CGG, AGG, UGG and GGG (NGG codons) and abortive translation by peptidyl-tRNA drop-off.
- Position dependent influences of Shine-Dalgarno and SD-like sequences on gene expression.

6. Results and discussion

6.1. A codon window in mRNA downstream of the initiation codon where NGG codons give strongly reduced gene expression in *Escherichia coli* (Article I)

The downstream region after the AUG initiator codon has been analyzed in previous work (Gualerzi *et al.*, 2000). Although non-interaction between the downstream region and the 16S rRNA is well documented, other data have shown that the nature of codons within this region could regulate gene expression in

Escherichia coli (Looman *et al*., 1987; Stenström *et al.*, 2001a; Stenström *et al.*, 2001b; Stenström and Isaksson, 2002).

Translational enhancer sequences upstream of the AUG initiation codons have been described (McCarthy and Brimacombe, 1994). The regulation of gene expression in the bacterium *Escherichia coli* was studied by changing the codon composition downstream after the AUG initiator codon. Most of the codons have been analyzed for their effects on gene expression at positions +2 to +7 using both a *lacZ* expression system and a 3A' test gene (Gonzalez de Valdivia and Isaksson, 2004; Stenström *et al*., 2001a). The results presented here show that most of the codons at position +2 influenced protein expression negatively, but once these codons were positioned at +3, +5, +7, the gene expression was increased at the level of translation (Gonzalez de Valdivia and Isaksson, 2004).

However, NGG codons kept the total gene expression of the β-galactosidase enzyme from positions +2 to +5 at low levels. For this effect to occur NGG codons must be in the correct reading frame. Moreover, when NGG codons were positioned further down from the downstream region, the gene expression was increased, regardless of the codon composition itself. Other codons with 2G nucleotides like GGN and GNG did not lower gene expression.

Polarity effect

The *lacZ* gene carries internal transcription polarity signals that possibly could trigger premature termination of transcription, thus giving a reduced level of gene expression. Such signals are not present in the semi-synthetic 3A' gene (Jin *et al.*, 2002). The position dependent negative effect by NGG, as found also in the 3A' test system therefore indicates that the codon effect on gene expression is not the result of an abortive event like transcriptional polarity. The similar results obtained on gene expression by NGG codons in the DR, using two different model genes, suggest a general effect on gene expression by NGG in the early coding sequence of mRNA rather than being connected with the choice of test gene.

mRNA secondary structure

Lowered gene expression by +2 codons could not be explained by involvement in mRNA secondary structures (Stenström *et al.*, 2001a; Stenström *et al.*, 2001b). Using gene variants examined here, computer analysis was done for secondary structures (Zuker *et al.*, 1999) involving a nucleotide sequence of about 20 nucleotides flanking the initiation codon on each side. Such analysis did not indicate any differences in mRNA secondary structures, which could explain the observed effects, notably by the early NGG codons that give low gene expression as described above.

mRNA degradation

All the gene variants analyzed in this study have the same promotor region. Therefore, the frequencies of transcription initiation should be similar. However, the effects on mRNA degradation by the altered DR-A codons are not known making it conceivable that the observed low gene expression values in the presence of certain codons could be the result of a decreased mRNA pool. This is, however, not the case since our estimates of mRNA levels for AGG gene variants, using the 3A' system with 2A' as an internal control, suggest that the relative mRNA levels are similar for AGG at positions +5 and +7 as well as for AGA at these locations.

6.2. Abortive translation caused by peptidyl-tRNA drop-off at NGG codons in the early coding region of mRNA (Article II)

The processivity error known as drop-off was reported in 1972 (Atherly and Menninger, 1972). According to Kurland (Kurland, 1992) drop-off is the main processivity error in *Escherichia coli.* In the present work, we tested the NGG constructs reported in paper I.

To do this test for excessive drop-off of peptidyl-tRNA at certain early codons (including the NGG codons) they were placed in the 5' coding region of a *lacZ*

reporter gene in a plasmid and introduced into the *pth*(Ts) mutant strain MB01, with its temperature sensitive peptidyl-tRNA hydrolase. It was found that the low gene expression associated with NGG codons in the downstream region following the initiation codon is the result of peptidyl-tRNA drop-off, that is excessive enough to inhibit growth of a *pth*(Ts) mutant strain (Gonzalez de Valdivia and Isaksson, 2004; Stenström *et al*., 2001a).

Extra tRNA

Overexpression of certain cognate tRNAs can give an increased gene expression (Brinkmann *et al*., 1989; Imamura *et al*., 1999; Menez *et al.*, 2000; O'Connor, 1998; Sorensen *et al*., 2003; Zahn, 1996). However, overexpressed tRNAs could be unmodified, and therefore less efficient in translation (Björk, 1996; Gustafsson *et al.*, 2004).

Here, we have used the 3A' reporter gene system to analyse if the low gene expression and the inhibitory effect on growth of the peptidyl-tRNA hydrolase strain associated with some early codons could be rescued by over-expression of the cognate tRNA. However, we did not observe any compensation of low gene expression by over-expressed tRNA at least for the analysed NGG codons, AGG and GGG. This is true either if the tRNA is cognate to the codon before or to the particular NGG codon itself, as analysed here.

Transfer-messenger RNA (tmRNA)

tmRNA is a very interesting small stable RNA that can be found in all eubacteria as well as in some chloroplasts and mitochondria (Ivanov *et al.*, 2002; Shpanchenko *et al*., 2005). It is also known as 10 Sa RNA or SsrA RNA, and is unique because it combines the activities of both tRNA and mRNA in a single molecule (Withey and Friedman, 2002). The primary function of tmRNA is the release of stalled ribosomes from truncated mRNA that lacks stop codon (Ivanov *et al.*, 2002; Withey and Friedman, 2002).

We used a strain lacking the tmRNA gene (K8619-*Ssra::cat*) and the 3A' reporter gene to test for any involvement of tmRNA in the low gene expression caused by an early NGG codon. The NGG constructs that give very low gene expression were transformed into strain K8619 and gene expression was checked. The results did not show any increase in gene expression compared with the wild type strain with tmRNA (data not shown). Therefore, we could assume that the low gene expression associated with the NGG codons was not the result of tmRNA activity.

Translation of early codons

It could be speculated that during early translation the length of the nascent peptide is too short to reach the protein exit tunnel through the 50S subunit (Dinçbas *et al.*, 1999; Karimi *et al.*, 1998; Yonath and Berkovitch-Yellin, 1993). However, even though we find lowered expression in the cases of the arginine codons CGG and AGG, such an effect was not observed for the other analyzed arginine codons AGA, CGC, CGA and CGU.

The amino acid sequence of the nascent peptide is the same in all these cases. This eliminates any contribution by the amino acid residues in the nascent peptide to the observed low expression values observed for the NGG arginine codons. Similar arguments apply to the glycine codon family at positions +2 to +5 with GGG giving low expression, while the others give high expression (Gonzalez de Valdivia and Isaksson, 2004). We suggest a model implying that the codon/anticodon interaction involving NGG codons is intrinsically weak in the early coding region, thus frequently leading to an abortive event like peptidyl-tRNA drop-off.

6.3. Influences on gene expression *in vivo* by a Shine-Dalgarno sequence (Article III)

In bacteria, the canonical Shine-Dalgarno sequence is base pairing with 16S rRNA between the head and the platform of the ribosome. Other types of base pairing

between mRNA and 16S rRNA have also been suggested (Petersen *et al.*, 1988; Sprengart *et al.*, 1996; Thanaraj and Pandit, 1989). No RNA complementarity between the region downstream of the AUG initiator codon and the 16S rRNA sequences has been found (Moll *et al.*, 2001; O'Connor *et al.*, 1999; Tedin *et al.*, 1997).

However, the downstream region, due to the presence of a single codon, can affect the level of translation at the early coding region (Faxén *et al.*, 1991; Gonzalez de Valdivia and Isaksson, 2004; Stenström *et al.*, 2001a). In addition, different downstream regions (DRs) as such have been reported to influence the level of gene expression in *Escherichia coli* (Stenström *et al.*, 2001b). It appeared possible that some of these DRs provoked low gene expression because they could form a SD-like sequence. This finding compelled us to study the effect of SD-like sequences downstream of the AUG initiation codon.

SD-like sequence between two initiation start sites

The results confirm the negative effect on gene expression by a SD-like sequence downstream of an initiation codon. The negative effect provoked by the SD-like sequences is not due to frameshifting or mRNA degradation by ribonucleases (data not shown).

Although a SD-like sequence inhibits initiation at an upstream initiation codon, it apparently stimulates the initiation of translation of a second initiation site downstream of the SD-like sequence.

Data suggest that the ribosome can bind to a SD-like sequence situated between two start sites and initiate translation elongation at the site with better features for initiation. These two starts site can compete for this ribosome, depending on their efficiencies as start sites.

Stem-loop structure

A very stable stem-loop structure was introduced after the first initiation site upstream of a SD-like sequence and the second initiation site. In this case, the

translation initiation takes place exclusively at the second initiation site and the stem loop prevents upstream initiation. This result suggests that some additional constraint is associated with the stem-loop. The model implies that a postulated upstream diffusion along mRNA is prevented by the introduced stem-loop structure.

The upstream effect by a SD-like sequence is thus the result of a functional compromise between a negative and a positive effect. The negative effect is most likely the result of blocking binding of an upstream ribosome, and the positive effect could be the result of ribosome upstream diffusion along the mRNA starting from the downstream SD-like sequence.

Peptidyl-tRNA drop-off

As a possible cause of low gene expression in the presence of SD-like sequences downstream of the initiation codon peptidyl-tRNA drop-off was analyzed. A control plasmid pEG1216 known to give peptidyl-tRNA drop-off, and a MB01 mutant strain *pth*(Ts) unable to promote hydrolysis of the peptidyl-tRNA at 37°C were used. Although plasmid pEG1216 as expected inhibited growth of the *pth* mutant strain, the two plasmids carrying a SD^+ variant with the out of frame triplet AGG (AAGGAGG), or a SD^- showed no difference in growth inhibition at 37°C after IPTG induction. The results suggest that there is no excessive peptidyl-tRNA drop-off in the analyzed strain with a downstream SD^+ or SD^-. Different rates of peptidyl-tRNA drop-off do not seem to be the reason for the negative effect by the downstream SD-like sequences on gene expression.

mRNA secondary structure

The secondary structure of mRNA close to the initiation codon can affect translational initiation efficiency. Secondary structures of the SD^+ and SD^- gene variants were analyzed. The analysis suggested that the secondary structure of the initiation regions of the mRNAs are similar (data not shown). It is less likely that interference on translation initiation in the presence of downstream SD^+ or SD^-

sequences due to mRNA secondary structure could be an explanation for the different levels of gene expression.

Using a model of Shine-Dalgarno sequences (Shultzaberger *et al.*, 2001) a computer search was made for genes carrying a SD+ or SD-like sequence among the first 20 codons for all genes annotated in EcoGene 20 in *Escherichia coli* K12 genome. A few cases were found where two potential initiation codons were located at an appropriate distance from an SD-like sequence between them. However, protein BLAST searches suggested that also for these cases, the second initiation codon is the annotated start site, i.e. there is an SD sequence upstream, but not downstream, of the start site. The data point out that for natural genes the SD^+ or SD-like sequences are systematically avoided in the early coding region of mRNA.

Our data suggest that ribosomes anchor to the SD^+ sequence irrespective of its location relative to the initiation codon. Therefore, the location of SD^+ or SD-like sequences can strongly influence gene expression at the translational level. This should provide a strong evolutionary constraint for codon usage, especially in the early coding region in natural genes.

7. Concluding remarks and future work

We have presented several results concerning mRNA sequences around the canonical initiation codon. These sequences downstream or upstream of the start codon strongly influence the translation process and gene expression in *Escherichia coli*. This work has clearly shown how a choice of a single codon from a codon family that code for the same amino acid, could influence up-down gene expression if located within the downstream region (from +2 to +5) after AUG (+1).

The down regulation of the gene expression by a single codon is only seen at the very beginning of the coding region. We suggest that the low gene expression caused by NGG codons is provoked by peptidyl-tRNA drop-off due to a weak interaction between codon and the corresponding anticodon. In this particular case of down regulation of the gene expression by a single codon, we did not see any involvement of mRNA secondary structure, polarity effect, tRNA and mRNA concentration, analyzed one by one.

The upstream sequence known as SD/SD-like sequences have a main role in translation initiation in *Escherichia coli*. The SD/SD-like sequences not only ensures an effective translation initiation, but also helps the ribosome to find the most suitable region for translation if it is situated between two initiation sites. Although downstream SD/SD-like sequences have a negative effect on upstream initiation, in the absence of strong downstream start site, the SD/SD-like sequence anchored ribosome can initiate translation at an upstream start site. However, in general, SD-like sequences downstream of the initiation codon should be strongly avoided in high expressed genes in *Escherichia coli* as found here.

This study has shown that the mRNA sequences surrounding the initiation codon (the downstream region, the Shine-Dalgarno and SD-like sequences) can influence

translation and gene expression. These data should be considered in works related to recombinant protein expression using *Escherichia coli* as a host.

Further studies

Hence, we feel that this study on translation and gene expression should be extended to natural polycistronic mRNA genes in *Escherichia coli* in order to investigate their impact on the physiology of Bacteria.

We need to continue analyzing the influence of the 5' UTR region itself on translation and with respect to the S1 binding site sequence in the presence of weak Shine-Dalgarno or non-functional Shine-Dalgarno sequences.

The mechanism behind the drop-off connected with NGG codons is not clear yet. Therefore, we should consider doing *in vitro* studies, which can give us some insight of the possible mechanism that cause abortive translation within the DR. In addition, there are some promising results linking drop-off at the beginning of the gene and antibiotic resistance in *Escherichia coli* that need to be followed up.

Many interesting studies can be done in the field of ribosome and translation initiation in the years to come. The new ribosome structure showing the translation process at the atomic level will answer exciting questions about the drop-off at the early coding region; it will clear the role and the position of the initiation factors. In addition, it can provide better understanding on how the process of initiation is for leaderless and leadered mRNAs and it would define the importance of ribosome, proteins and the rRNA in the translation process, knowing that the ribosome is a ribozyme.

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