

Received 4 December 2002
Accepted 20 March 2003

Short Communication

**THE OPTIMAL EUKARYOTIC SIGNAL FOR TRANSLATION
INITIATION FROM NON-AUG CODONS, PRESENT UPSTREAM OF
BACTERIOPHAGE λ P CISTRON, IS INACTIVE IN *Escherichia Coli***

BORYS WRÓBEL^{1,*}, BARTOSZ SŁOMIŃSKI²
and GRZEGORZ WĘGRZYN^{1,2}

¹Institute of Oceanology, Polish Academy of Sciences, Św. Wojciecha 5, 81-347
Gdynia, Poland, ²Department of Molecular Biology, University of Gdańsk,
Kładki 24, 80-822 Gdańsk, Poland

Abstract: Expression of the replication genes of bacteriophage λ , *O* and *P*, is believed to be translationally coupled. However, it was previously noted that, under conditions of amino acid starvation, when *O* is not synthesized, *P* continues to be expressed at a relatively high level. The results presented in this report, contrary to the previously presented hypothesis, suggest that an AGACUGGAU sequence (an optimal context for translation initiation from non-AUG codons in eukaryotes, and present upstream the *P* cistron) is inactive in *Escherichia coli*. Comparative sequence analysis confirms that such a signal is unlikely to be important for *P* synthesis. Instead, a weak Shine-Dalgarno sequence may be present upstream the *P* cistron, and be active in the absence of *O* gene expression.

Key Words: Bacteriophage λ , Translational Coupling, Translation Reinitiation, S-gal (3,4-cyclohexenoesculetin- β -D-galacto-pyranoside)

INTRODUCTION

It was previously noted that in amino acid-starved *Escherichia coli* cells carrying a λ plasmid (a plasmid utilizing the bacteriophage λ replication module), λ P protein is preferentially synthesized [1]. The λ P gene follows *O* (Fig. 1), and both genes code for λ replication proteins. The Shine-Dalgarno sequence in front of λ *O* (AACAGGA) is close to optimal (AGGAGGA), as is its distance (6 nt) from the ATG codon [2]. The stop codon of *O* (UGA) overlaps with the start

*Corresponding author, Phone: (+48 58) 301 2241 ext. 377, Fax: (+48 58) 301 0072,
E-mail: wrobel@biotech.univ.gda.pl

CCAGA CCAACTGG). The PCR fragment was digested with *Xba*I and *Sph*I, and ligated to the vector pBAD24 [5], previously digested with the same enzymes. In the pBI0 plasmid thus created, several sites for enzymes that leave the same overhangs are present upstream of the *lacZ* sequence. This allowed for the creation of plasmids with different sequences preceding the start codon by digesting pBI0 with appropriate enzymes and religation: pBI1 (*Nhe*I-*Xba*I deletion and religation), pBI3 (*Nhe*I-*Spe*I), and pBI6 (*Avr*II-*Spe*I). The plasmid pBI7 was created by *Nhe*I-*Xba*I deletion and religation of pBI6. pBI10 was created as pBI0, but by first amplifying the *lacZ* gene using the primers lacZL1 (5'-GGGGGGCTAGCAGACTGGATA CCGCGGATTCCTGGCCGT) and lacZR. The structure of all the plasmids was confirmed by restriction analysis and sequencing. *E. coli* K-12 strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^qZΔM15 Tn10(Tet^R)*]) [6] was used in all the assays.

The activity of *lacZ* fusions was tested on S-gal (3,4-cyclohexenoesculetin- β -D-galacto-pyranoside) [7] LB agar plates (Sigma-Aldrich) without arabinose (with activation, the observed differences in gene expression were less pronounced; data not shown), and in liquid cultures using a β -galactosidase assay as first described by Miller [8]. However, we found that although the bacteria carrying plasmid pBI7 grew black on S-gal plates (and blue on X-gal plates), no β -galactosidase activity could be detected when liquid cultures were used in the classical Miller's assay, even in the presence of arabinose (data not shown). Therefore, in order to quantify the LacZ activity, we developed an S-gal based assay. 0.2 OD units of exponentially growing bacteria were spread on 10 cm Petri dishes with 20 ml of S-gal LB agar (Sigma-Aldrich). After 16h growth at 37°C, 1 ml of S1 buffer of the Qiagen Miniprep kit (Qiagen) was sprinkled on each plate and the cells were collected. After ensuring that an equal cell mass had been collected, 0.5 ml of lysis solution S2 from the same kit was added to 0.5 ml of the suspension. Then, the cell debris was removed by centrifugation, the supernatant was diluted five times, and its absorbance measured at 380 nm. The plasmid DNA levels in bacteria carrying various constructs did not differ significantly (data not shown). This was ensured by densitometry (using Fluor-S-MultiImager, Bio-Rad) after alkaline lysis, linearization by a restriction enzyme and agarose gel electrophoresis.

RESULTS AND DISCUSSION

Table 1 shows the results of the measurement of β -galactosidase activity using the S-gal assay for bacteria carrying plasmids of the pBI series. It can be noted that the presence of the sequence AGACTGGAT allows activity in strains carrying pBI7, but when the ATG codon downstream is absent in pBI10, the gene is inactive. This suggests that, contrary to previous speculations [1], the AGACUGGAU sequence acts at best as a weak Shine-Dalgarno sequence (in the

artificial context of pBI7), and not as an eukaryotic-like signal for the initiation of translation from non-AUG codons.

The question remains, however, as to what translational signal is used to bring about preferential synthesis of the P protein in the absence of *O* translation (it must be added that such a signal would be expected to be conserved only if such expression were adaptive). A comparative analysis of the related, but not identical, sequences might be helpful in finding the answer. Two such sequences, of phages $\phi 21$ and $\phi 80$, are present in the public databases. Both phages show genetic organization of the replication/regulation module identical to that of λ , and can be expected to share similar regulation signals. Although the sequences themselves are not identical, their

Tab. 1. The activity of *lacZ* fusions to translation signals carried on pBI plasmids. OD_{380} was measured as described in the text relative to pBAD24 (empty vector) in three independent experiments. The mean \pm squared standard deviation is shown. Shine-Dalgarno and AGACTGGAT sequences are in bold. For pBI3 and pBI7, the underscores indicate the extent of deletions in comparison with pBI1.

Plasmid	5' end of mRNA	OD_{380}
pBI1	acccgTTTTTTgggct agactggat cctaggaggactagt atg gatt	1.2 \pm 0.1
pBI3	acccgTTTTTTggg_____ctagt atg gatt	0.01 \pm 0.02
pBI7	acccgTTTTTTgggct agactggat c_____ctagt atg gatt	0.40 \pm 0.06
pBI10	acccgTTTTTTgggctagc agactggat accgcggattcactggccgt	0.025 \pm 0.02

similarity is very high. Therefore, one can only be certain which parts of the sequence are NOT conserved. The ANN---GAU sequence is apparently not (Fig. 1), which can be interpreted as confirming the conclusion that CTG in the context of an eukaryotic-like signal is not used to bring expression of *P* when *O* is not produced. However, it can be noted that in the region coding for Tyr, Gly and Val, although all three codons are fully degenerated, only one difference is observed (in the $\phi 80$ sequence). This sequence (AC/TGGGG) might potentially function as a Shine-Dalgarno sequence (consensus AGGAGG [2]). After this region, the end of the reading frame is again not conserved.

Although the results of this study allow us to dismiss the hypothesis of the activity of the AGACUGGAU sequence in prokaryotes, neither the results of the comparative sequence analysis nor those obtained with the artificial translation signals can be expected to be equivalent to direct experimental evidence

confirming the activity of a potential Shine-Dalgarno signal upstream *P*. We might only speculate that such a signal might be active in the absence of the expression of *O* and translational coupling, as was recently observed in another system [9]. We may note that further experiments using a similar system of plasmids, might help to investigate under what conditions upstream translation is inhibitory for the expression of a downstream cistron. This problem also has biotechnological implications, as translational coupling is sometimes used to increase the level of expression [10].

We may also note that, using the classic Miller's assay [8], we could not observe LacZ activity in strains carrying pBI7 and pBI10, even after induction with arabinose. Perhaps the conditions of growth in colonies allow for the accumulation of the LacZ protein (or the product of its activity) and thus the detection of gene expression. Such accumulation would not be observed in the Miller's assay, where prolonged incubation leads to high background levels. If so, the values presented in Table 1 are not fully proportional to the levels of *lacZ* expression. However, the assay developed here might be helpful in detecting the activity of weak signals.

Acknowledgements. This work was supported by the Polish State Committee for Scientific Research (project No. 3 P04A 049 24). G.W. also wishes to acknowledge financial support from the Foundation for Polish Science (subsidy No. 14/2000).

REFERENCES

1. Obuchowski, M. and Wegrzyn, G. Synthesis of bacteriophage λ P protein in amino acid-starved *Escherichia coli* cells. **Biochem. Biophys. Res. Commun.** 222 (1996) 612-618.
2. Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G.D. and Gold, L. Translation initiation in *Escherichia coli*: sequences within the ribosome-binding site. **Mol. Microbiol.** 6 (1992) 1219-1229.
3. Andre, A., Puca, A., Sansone, F., Brandi, A., Anitco, G. and Calogero, R.A. Reinitiation of protein synthesis in *Escherichia coli* can be induced by mRNA cis-elements unrelated to canonical translational initiation signals. **FEBS Lett.** 468 (2000) 73-78.
4. Grunert, S. and Jackson, R.J. The immediate downstream codon strongly influences the efficiency of utilization of eukaryotic translation initiation codons. **EMBO J.** 13 (1994) 3618-3630.
5. Guzman, L.-M., Belin, D., Carson, M.J. and Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose *p*_{BAD} promoter. **J. Bacteriol.** 177 (1995) 4121-4130.
6. Bullock, W.O., Fernandez, J.M. and Short, J.M. XL1-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. **BioTechniques** 5 (1987) 376-378.

7. Heuermann, K. and Cosgrove, J. S-Gal: An autoclavable dye for color selection of cloned DNA inserts. **BioTechniques** 30 (2001) 1142-1147.
8. Miller, J.H. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972.
9. Yu, J.S., Madison-Antenucci, S. and Steege D.A. Translation at higher than an optimal level interferes with coupling at an intercistronic junction. **Mol. Microbiol.** 42 (2001) 821-834.
10. Mashko, S.V., Veiko, V.P., Lapidus, A.L., Lebedeva, M.J., Mochulsky, A.V., Shechter, I.I., Trukhan, M.E., Ratmanova, K.J., Rebentish, B.A., Kaluzhsky, V.E. et al. TGATG vector: a new expression system for cloned foreign genes in *Escherichia coli* cells. **Gene** 30 (1990) 121-126.