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Communication

Use of transcriptional slippage for diverse gene expression*

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We present here an alternative for two-promoter systems ensuring highly diverse expression of several genes from a single promoter. This approach assumes an introduction of a deletion mutation into an A/T homopolymeric run in a gene's proximal part, and employs the transcriptional slippage mechanism for insertion-dependent reinstatement of the proper reading frame by the T7 RNA polymerase.

Keywords: GFP reporter, expression system, InDel error, M2.MboII methyltransferase, T7 bacteriophage RNA polymerase, transcriptional slippage

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***This paper is dedicated to Professor Wacław Tadeusz Szybalski on the 100th anniversary of his birth**

Abbreviations: FS, frameshifted variants; InDel, either insertion or deletion of a nucleotide; WT, wild-type inframe variants

During the transcription process, in many regions of long homopolymeric poly(A) or poly(T) sequences a phenomenon of insertion or deletion of one or more nucleotides might occur (*transcriptional slippage*, Atkins *et al*., 2016). Programmed transcriptional slippage always occurs in well-defined homopolymeric sequences of some genes and contributes to the formation of an additional alternative protein that is essential for the cell function. This happens with high efficiency, and has a specific one-directional effect (either insertion or deletion [InDel] of a nucleotide), which is often ensured by the presence of additional second-order DNA structures that are necessary to make this process more likely (Penno *et al*., 2015). In case of some groups of genes (e.g. IS transposases) slippage is absolutely necessary for formation of the correct and functional protein (Baranov *et al*., 2005; Liu *et al*., 2018). The nature of the non-programmed transcriptional slippage is different. It is mainly related to the intrinsic property of the RNA polymerase (RNAP), which under certain conditions (long A/T homopolymeric sequences) may introduce random and two-directional InDel errors, contributing to an increase in mRNA polymorphism of a given gene (Larsen *et al*., 2000; Tamas *et al*., 2008; Wagner *et al*., 1990; Wons *et al*., 2015), with changes in the primary reading frame (InDel frameshift). This leads to production of altered forms of proteins (Schwarz *et al*., 2021; Wons *et al*., 2015; Rockah-Shmuel *et al*., 2013). Such a mechanism turns out to be beneficial in case of genes with a single InDel mutation, as it can "repair" the mRNA sequence (including reinstatement of the correct reading frame) while keeping the gene's DNA sequence mutated (Gordon *et al*., 2013; Koscielniak *et al*., 2018; Rockah-Shmuel *et al*., 2013; Tamas *et al*., 2008; Wernegreen *et al*., 2010; Wons *et al*., 2015).

Many methods based on various protein overproduction systems are focused on efficient expression of a desired gene that assumes fidelity and reproducibility of this process. In the case of co-expression, employment of two different expression systems controlled independently is most widely used, leading to varied co-expressed protein proportions. Alternatively, usage of the same type of a promoter results in a balanced and equal level of both genes' expression (Novy *et al*., 2002). Here, we propose a novel approach to achieve a highly different expression level of two or more genes from a single controllable promoter. The method presented takes advantage of a very effective and commonly used bacteriophage T7 expression system. A reduction in protein production is achieved by introduction of a single nucleotide deletion (frameshift mutation) within the polyA/T sequence of the proximal part of a given gene. Reinstatement of the proper reading frame is insertion-dependent and employs transcriptional slippage mechanism of the T7 RNAP. This enzyme is highly capable of transcriptional slippage on such sequences, especially by nucleotide insertion (Koscielniak *et al*., 2018; Wons *et al*., 2015; Wons *et al*., 2018), and what is important, in contrast to the host polymerase, T7 RNAP generated expression manifests its intrinsic resistance to transcriptional polarity in the absence of T7-specific terminators (Chevrier-Miller *et al*., 1990). Feasibility of the proposed approach is demonstrated here with combination of tandem genes: *mboIIM2* and *gfp* (transcriptional fusion, each gene possesses its own Shine-Dalgarno sequence), encoding the DNA methyltransferase M2.MboII and GFP proteins, respectively, both as inframe (WT) and frameshifted (FS) variants (Fig. 1). The four combinations of tandem genes were introduced into the pET24a plasmid vector (Novagene) and were constructed as follows: WT or FS *gfp* gens were subcloned from pBADmingfpA$_6$0 and pBADmingfpA$_5$-1 as EcoRI-digested DNA fragments (Wons *et al*., 2018) and inserted into the EcoRI site of pETmboIIMB.3 (carrying *mboIIM2* WT gene, Furmanek-Blaszk *et al*., 2009) or pETmboIIM2FS vectors (site-specific mutagenized variant of *mboIIM2* with deletion of the 18th thymine residue, using the PfuPlus DNA polymerase in a PCR reaction – Eurx-Gdansk, Poland) (Fig. 1). All plasmids were introduced into *Escherichia coli* ER2566 strain which hosts the T7 RNA polymerase (NEB Ipswich, USA). After 1 h induction with 1 mM IPTG, the effects of diverse levels of expression were assayed both, qualitatively and quantitatively (at least in the case of GFP protein) (Fig. 2). For GFP analysis, measurement of cell fluorescence and immunodetection of the protein by western blotting were used (Wons *et al*., 2018). For M2.MboII, an *in vivo* methylation test was utilized (DNA protection against cognate MboII endonuclease cleavage)

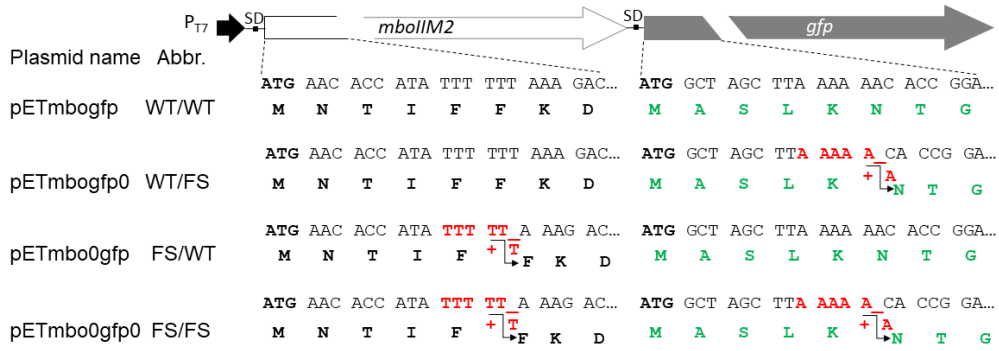

Figure 1. Details of the relevant sequence of the *mboIIM2* and *gfp* transcriptional fusion genes, in four tandem combinations including the wild-type (WT) and frameshifted variants (FS, single nucleotide deletion).
The plasmid names, abbreviations of the gene combinations, SD (Shine-Dalgarno) sequence and amino acids residues are shown. The location of the A/T homopolymeric runs (bold, in red) deletion sites are marked as a line in red. The likely site of nucleotide insertion (either +A or +T) in homopolymer runs after slippage is marked by an arrow.

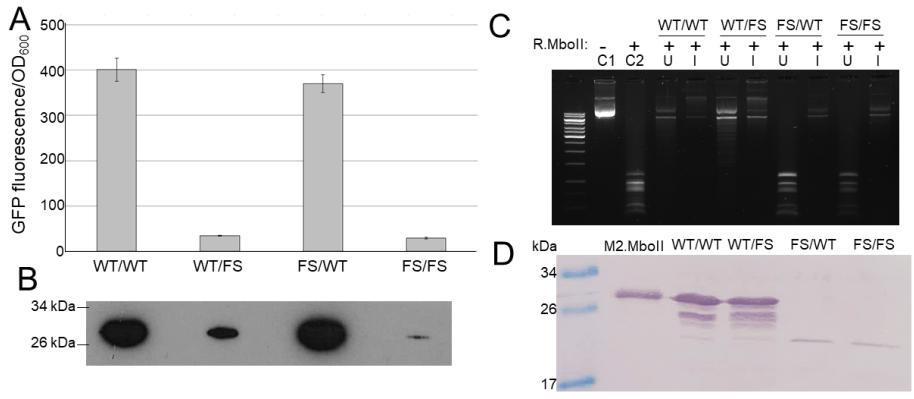

Figure 2. Expression levels of the four combinations of *mboIIM2* and *gfp* gene variants.
(**A**) Relative GFP activity shown in fluorescence units normalized to OD_{600} of the bacterial culture. Error bars represent standard deviation from at least five independent experiments. (**B**) Expression levels of the corresponding *gfp* constructs by western blotting and immunodetection of GFP. (**C**) Relative level of plasmid DNA methylation by M2.MboII challenged by R.MboII endonuclease digestion. (-/+) denotes untreated and R.MboII digested DNAs, respectively: C1 – untreated pETmbo0gfp0; C2 – digested unmethylated pETgfp; U – plasmids from uninduced bacteria; I – plasmids from induced bacteria (1 mM IPTG by 1h). (**D**) Expression levels of the corresponding *mboIIM2* constructs assessed by western blotting and immunodetection of M2.MboII after 1h induction with 1 mM IPTG.

and immunodetection was determined by western blotting (Wons *et al.*, 2015). Quantification of GFP fluorescence of a 400 µl cell culture sample (Varioskan Flash Spectral Scanning Multimode Reader spectrophotometer – Thermo Scientific, with excitation and emission wavelengths of 488 and 510 nm, respectively) (Fig. 2A) is proportional to immunodetected GFP products (mouse monoclonal anti-GFP (B-2) antibodies – Santa Cruz Biotechnology, Fig. 2B) (Wons *et al.*, 2018). As can be seen, low level of *gfp* expression was obtained even when FS *gfp* variant was employed, with no effect of the upstream *mboIIM2* gene's status. Similarly, M2.MboII production was immunodetected (Fig. 2D, rabbit polyclonal anti-M2.MboII) and the enzyme's activity was determined as follows: plasmid DNA with 18 MboII sites was isolated from bacterial cells carrying appropriate expression plasmids and then treated with cognate MboII restriction endonuclease (Wons *et al.*, 2015) (Fig. 2C). All plasmids from induced cells were protected against endonuclease cleavage, even though M2.MboII was not detected by immunodetection in the FS *mboIIM2* samples. Again, this demonstrates that a low level of WT *mboIIM2* expression took place even in the FS variant and this was not affected by the *gfp* gene's status.

In summary, we demonstrate here that transcriptional slippage is a suitable tool for restoring the WT reading frame in a gene containing a frameshift mutation and obtaining a low level of its expression, while the tandem WT/WT variants retain a high level of expression. This system can be used wherever the production of a valuable molecular poison can only take place in the presence of an antidote that must be removed during purification, which always increases the steps in detection and control procedures.

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