

Changing the transcriptional activity of genome regulatory loci by PCR-mutagenesis

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Transcription requires the presence of a single promoter upstream of a gene, but in many cases there are multiple transcription signals. Promoter clustering is common for rRNAs genes^[1], plant mitochondrial genes^[2], being quite a widely distributed phenomenon for vertebrate genomes, including the human^[3]. It is also inherent to bacteria and, in particular, to *E. coli*. This clustering provides an additional possibility for adaptive regulation in different growth conditions and in different physiological processes^[1-5]. Although the number of independently functioning promoters in the cluster vary in the range 2-8 (exemplified in Fig. 1, left panel), in most cases 2 or 3 transcription signals are sufficient for adequate gene expression. Hence it was highly unexpected to find 434 loci in the genome of *E. coli* with extremely high density of promoter-like sites (≥ 8 signals in 100bp frame along 300 bp), predicted by promoter finder PlatProm (Fig. 1, right panel). We called them “promoter islands” (PIs)^[6,7]. Seventy-eight of them are composed of promoters, recognized by σ^{70} -RNA-polymerase, while the remaining “islands” include promoters with different σ -specificity^[7].

Promoter clusters usually show higher transcriptional activity than single promoters^[8]. However, “islands”, on the contrary, demonstrated very low propensity to initiate synthesis of full-sized transcripts. It was found that 85% of PIs are associated with foreign genes originated in the *E. coli* chromosome due to horizontal transfer from other bacteria^[7,9]. Low transcriptional activity of PIs may therefore be evolutionary adapted to arrest expression of foreign genes, i.e. to perform a function that is not typical for the promoter regions.

The aim of this study was to estimate the changes occurring in the promoter distribution profile in comparison to a normal promoter, in the course of random mutagenesis with subsequent picking out up- and down-regulated mutated promoter derivatives. If RNA polymerase binding to multiple promoters prevents effective transcription, up-regulation in this model system should be accompanied by a decrease, rather than increase in the number of potential promoters.

Two chromosomal loci were chosen for the experiment – a PI associated with the *appY* gene and a regulatory region of the *dps* gene, containing several functional promoters^[10] (Fig. 1).

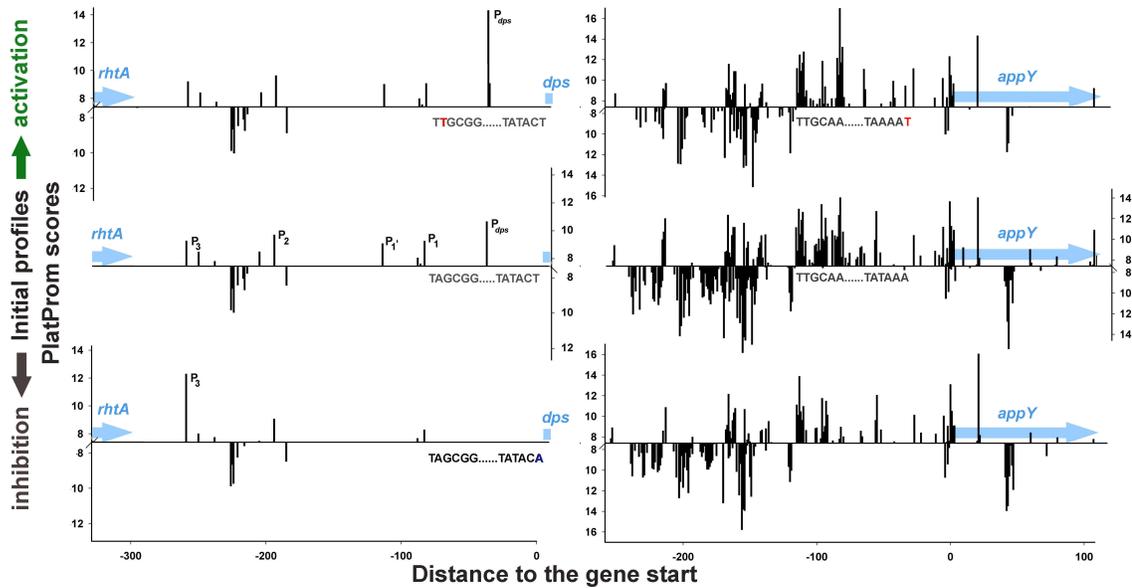


Fig. 1. Disposition of potential promoters in the regulatory region of *dps* (left) and “promoter island” of *appY* (right). Vertical bars above and below the X-axis show the predicted TSPs for the upper and lower strands, respectively. The height of the peaks reflects promoter score, calculated by PlatProm. Blue arrows denote the *E.coli* genes. Two central panels show PlatProm scans obtained for natural genomic sequences. Two upper and two lower panels demonstrate changes obtained upon selection of derivatives with higher and lower promoter activity, respectively. Mutations affected contexts of consensus promoter elements -35 (*dps*) and -10 (*dps* and *appY*) are indicated.

The experimental scheme is shown in Fig. 2. The first step includes PCR mutagenesis using GeneMorph II Random Mutagenesis Kit (Agilent Technologies), where approximately 1-5 ng of plasmid containing the fragment to be mutated is used as a template in amplification. The PCR program included 2 minutes preheating at 95°C, then 30 cycles: 95°C 30 sec (DNA melting), 55°C 30 sec (primers annealing), 72°C 1 min (DNA synthesis). PCR-fragments were gel-purified by QIAquick® PCR Purification Kit (Qiagen, Germany) subjected to restriction with XbaI and BglII (1.5 h at 37°C) (Thermo Scientific, USA) and ligated with T4 ligase (overnight at +4°C) (Thermo Scientific, USA) into the pET28b plasmid in front of a promoterless *gfp*. *E. coli* Top10 cells were transformed with the derived vector using 0.2 M CaCl₂ and plated on LB agar plates supplemented with 80 mkg/ml kanamycin. Preliminary selection of the outgrown colonies was accomplished judging by the fluorescence level using a fluorescent microscope. The selected clones were then grown overnight at 37°C in liquid LB medium. The cells were harvested by centrifugation, resuspended in 10mM Tris-HCl (pH8.0), 1mM EDTA) and sonicated. Fluorescence intensity was measured for each supernatant sample individually and normalized on total protein concentration. Plasmids were extracted from cells with the highest and lowest fluorescence/protein ratio, sequenced and used in the next cycle of mutagenesis and sequencing.

Four rounds of PCR and negative selection accumulated 6 point mutations in the *dps* regulatory region, which decreased PlatProm scores for the main (P_{dps}) and three minor (P_1, P_1' and P_2) promoters. As a result transcriptional activity in this region was completely suppressed (left bottom panel in Fig. 1).

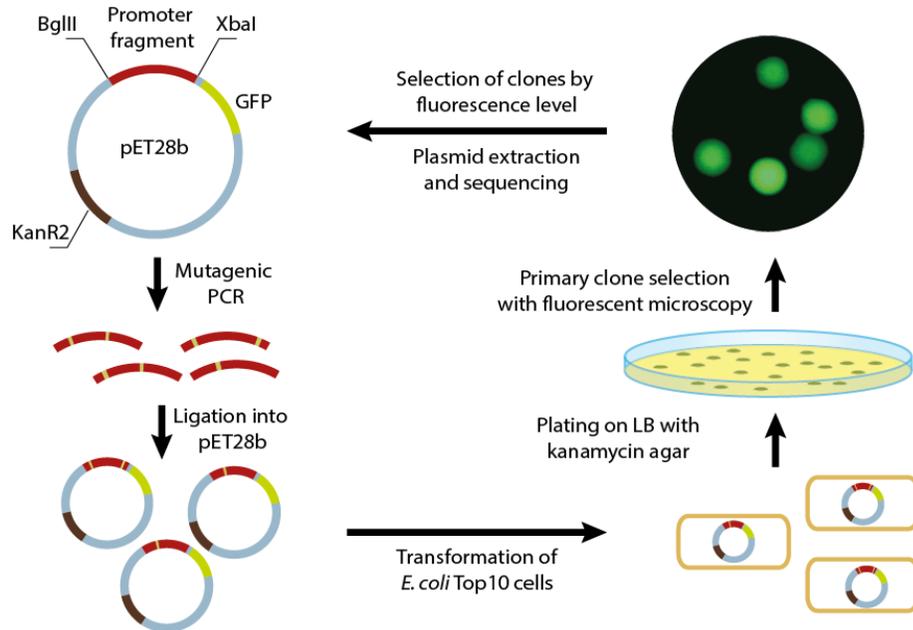


Fig. 2. Schematic layout of the SELEX procedure.

The *dps* promoter area selected for increased transcription contained 9 mutations (3 rounds), resulting in a 4-fold efficiency gain, with 12 possible RNA starts instead of 10 in the initial sequence (20% addition) and closer to consensus (TTGACA) substitution in the -35 element of P_{dps} (left top panel). Transcriptional activity of *appY*-associated PI was also suppressed to the background level upon four rounds of negative selection, which accumulated 11 point substitutions (right bottom panel). The amount of possible transcription start points (PSPs) decreased by 27%, though the fragment carrying the *appY* “promoter island” still contained 62 potential transcription signals on the top strand. Multiple promoters therefore can not guarantee active transcription. The positive selection for the “promoter island” of *appY* led to a 6.1-fold increase of the expression level (6 rounds). It was caused by 15 point substitutions and 3 single base pair deletions. These mutations also promote a decrease in the amount of possible TSPs from 85 to 63 and noticeable rise in the score of the promoter, located 85 base pairs upstream of the gene start (right top panel in Fig. 1). Thus, in case of *dps* promoter region the situation is very predictable – in positive selection the amount and scores of individual starts were increased, while in negative selection their quantity and quality dropped down. But in case of the *appY* PI both positive and negative selection decreased the

amount of possible TSPs. Confirming a hypothesis assuming obstacles for effective transcription initiation connected with a high density of potential promoters, these data also suppose instability of this high promoter density. It is possible that a special genomic system maintains the excessive amount of transcription starts in “promoter islands” so as to keep expression of neighboring genes quenched, but ready for rapid activation if necessary.

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