

BBSI Summer 2010

**Research Proposal:
Investigating the Sufficiency of Synthetic Upstream (UP) Elements to Initiate
Transcription in Prokaryotes**

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I. Introduction

The carefully controlled regulation of gene expression is critical to the proper function of both natural and synthetic gene networks. In naturally occurring prokaryotic genes and operons, transcription is initiated when a sigma-factor recognizes specific regions of the promoter, leading to the recruitment of the RNA polymerase (RNAP) core enzyme and the elongation of the mRNA transcript^{1,2}. The strength of a promoter (i.e., the rate of transcription) is controlled in large part by the affinity between the promoter sequence and the sigma-factor, with different sequences interacting with sigma-factors at varying intensity, leading to differing binding constants at transcription initiation¹. The classic example of this type of regulation is the binding of the σ^{70} or σ^{70} -related subunit of RNAP to the -10 and -35 hexamer sequences of the promoter^{1,2,3}. The variation in promoters' sequences gives the promoter, then, a natural control mechanism to regulate gene expression at the transcriptional level.

In the past two decades, however, more attention has been brought to the effects that non-promoter DNA has on regulating binding affinity to RNAP⁴. One example of this is a category of upstream (UP) elements – curved AT-rich regions of DNA that lie upstream of the sigma-binding regions of the promoter^{5,6}. UP elements range generally from -38 to -60 bases from the transcription start site and interact with the carboxyl-terminal domains of the two RNAP α subunits (Fig 1), significantly modifying genes' transcriptional activity compared with the promoters alone. One study that looked at the effects of various naturally occurring UP elements in *E. coli* found that transcriptional activity of various genes with both a promoter and an UP element ranged from 1.5 to 90 times the activity of transcription when only the promoter was present⁷.

Until recently, studies of UP elements have been limited to those that occur naturally. The idea of designing synthetic UP elements is attractive to synthetic biologists, however, as a finely tuned UP element can be used as another modular part to control a synthetic biological system. To this end, synthesized UP elements have been designed and characterized using qRT-PCR and gene expression assays involving *rffp*⁸.

Surprisingly, it has very recently been shown that synthetic UP elements can have much more significant effects on transcription than those that occur in nature. In an experiment using *E. coli* performed by Stephen Fong's laboratory at Virginia Commonwealth University, a synthetic UP element that was paired to an extremely weak promoter showed transcriptional activity indistinguishable from that of a normal promoter. The possible restorative effects of UP elements are therefore of great interest. The long-term question that we are interested in answering is: 1) are certain UP elements sufficient to initiate transcription in the absence of a promoter, and if not, 2) can we identify threshold in promoter activity that is necessary for the initiation of transcription?

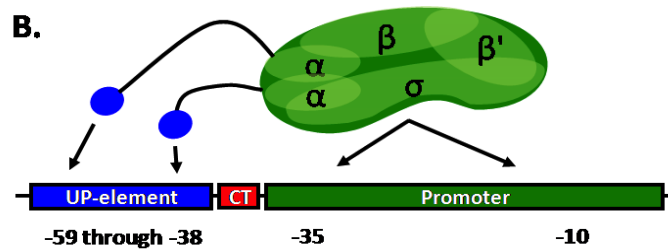


Fig 1

II. Goals for Summer 2010

By the end of the summer, I hope to have created and characterized the strengths of a series of promoters that provide a gradient of transcriptional activity from moderate to inactive (a.k.a. junk). Once these promoters are designed they will be synthesized and characterized using two assays. The first assay will be correlating transcriptional activity with the expression of the reporter gene *rfp* by measuring fluorescence using flow cytometry. The second assay will be a direct measurement of mRNA transcript levels by using qRT-PCR.

III. Methods

Promoter design

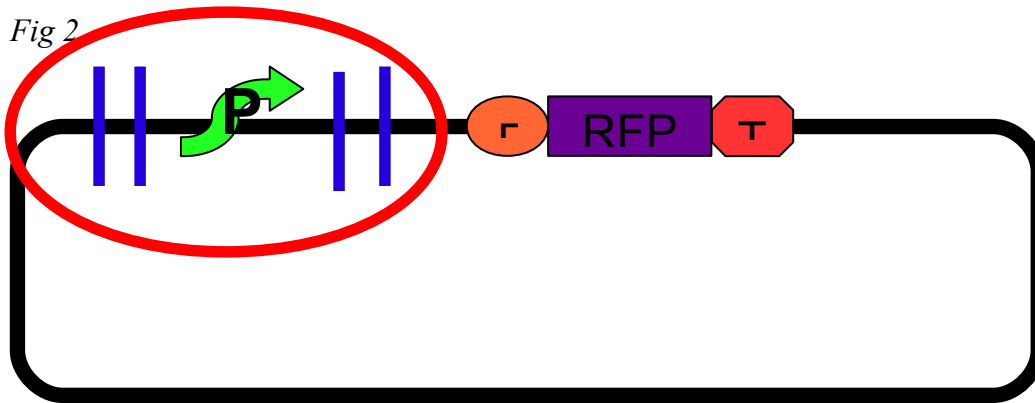
My tactic for designing promoters is that I will start with a standard promoter sequence of known activity and systematically sabotage it by altering individual bases in the -10 and -35 hexamer binding regions, resulting in a series of promoters that degrade in function due to loss of affinity at these conserved sites. My starting point will be a promoter called BBa_J23119 (sequence: ttgacagctagctcagtcctaggtataatgctagc), a constitutive promoter “consensus” sequence of strong basal activity that contains the canonical -10 and -35 hexamers (TATAAT and TTGACA, respectively) which characterize σ^{70} -family promoters. I will change individual bases in these regions (e.g., TAGAAT, TCTAAT, etc. in the -10 hexamer) and have these novel promoter designs synthesized by the DNA synthesis company IDT. I will also surround the sequences themselves with restriction enzymes to aid in designing the promoter-reporter construct.

Construct design

In order to use these promoters, they must somehow be arranged upstream of the reporter. *De novo* synthesis of the entire promoter-reporter construct is possible but would be very expensive and inefficient. Because the only variable in this study is the promoter itself, with the reporter sequence remaining constant, the optimal construct design is one where the unchanging components are reused, and only the variable portions are swapped out. One way of accomplishing this is with the use of restriction enzymes.

The *rfp* reporter will be present in a plasmid that contains restriction sites upstream of the coding region and some antibiotic resistance gene. These sites will match those surrounding the promoter sequence. This way the promoter can be effectively cut and ligated into the appropriate place on the plasmid (Fig 2). The specific method for assembling these various components has been described previously by Knight and colleagues⁹.

Fig 2



Promoter Activity Assays

In order to characterize these promoters, we need some way of assessing their ability to promote transcription. I will do this in two ways: fluorescence quantification using flow cytometry and mRNA quantification using qRT-PCR.

Each promoter that I design will drive the expression of the reporter gene *rfp*. By measuring the fluorescence of RFP protein (i.e., the amount of RFP), we can infer the amount of *rfp* mRNA, and therefore the amount of transcription. To perform this measurement, I will insert test plasmids in a strain of *E. coli* and grow the bacteria on LB plates containing an appropriate antibiotic. I will then suspend colonies in liquid LB and incubate them for a standard time in a shaker incubator. After this I will incubate them for three hours before removing samples to measure optical density and RFP fluorescence.

To measure mRNA transcripts of the *rfp* gene, I will follow a standard RNA isolation protocol and perform qRT-PCR, normalizing *rfp* mRNA levels to the endogenous level of *recA* mRNA

IV. Possible Results and their Implications

This project is designed to develop a method for testing the ability of synthetic UP elements to replace the lost function of canonical promoters in the genetic machinery of prokaryotes. By systematically targeting nucleotides in the -10 and -35 conserved regions, I will hope to have enough promoters that fall within the target range of function that will inform the future aspects of these studies.

Once this aspect of the project has been accomplished, it will be possible to compare the transcriptional activity of these promoters alone with their activity in cooperation with a synthetic UP element. There are two possible outcomes from this stage in the experiment. The first is that a strong UP element will restore transcription initiation at some threshold of promoter activity. From the characterization of each promoter in this experiment, this data can be used to better understand this minimum requirement for promoter activity, and therefore gain a better understanding of how UP elements function in the context of prokaryotic gene expression.

The second potential outcome is that a synthetic UP element will be able to restore gene expression to constructs whose promoters are equivalent to spacer regions. This would mean that an UP element is sufficient to initiate transcription, providing new insight into our current model of gene expression.

V. References

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