

# Analysis of the Effects of Synthetic Upstream (UP) Elements on Constitutive Promoter Strength

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## Abstract

Upstream (UP) elements are regions of DNA that usually lie between 40 and 60 bases upstream of the transcriptional start site in some prokaryotic genes and heavily influence the endogenous strength of the adjacent promoters in recruiting RNA polymerase (RNAP) by binding to the carboxyl tail domains (CTDs) of the RNAP alpha subunits. It has been shown previously that synthetic UP elements can restore transcriptional activity to promoters with basal strength indistinguishable from spacer regions. In this study, we attempted to investigate the sufficiency of synthetic UP elements to initiate transcription in *E. coli* by measuring adding the synthetic UP element K264006 to a random spacer sequence (Promoter J90001) the size of a normal constitutive promoter and measuring for transcription with an RFP reporter. Additionally we designed and characterized a library of weak promoters based on the J23119 promoter from the MIT Parts Registry that had heavily mutated -10 hexamer regions in an attempt to better understand the balance between the role of sigma70 and the alpha CTDs in recruiting the RNAP machinery. We found that the K264006 UP element was not sufficient to initiate transcription of the gene coding for RFP (Parts Registry #J06702). The promoters that were designed for this study all showed baseline fluorescence. UP elements need to be added to the promoters to see if any restoration of gene expression will take place.

## 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<sup>1,2</sup>. 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 initiation<sup>1</sup>. The classic example of this type of regulation is the binding of the  $\sigma^{70}$  or  $\sigma^{70}$ -related subunit of RNAP to the -10 and -35 hexamer sequences of the promoter<sup>1,2,3</sup>. 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 RNAP<sup>4</sup>. 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<sup>5,6</sup>. 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  $\alpha$  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 present<sup>7</sup>.

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 *rfp*<sup>8</sup>.

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?

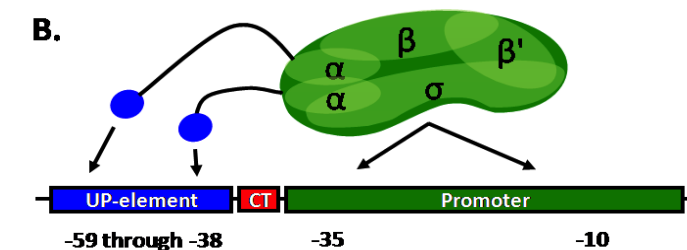


Fig. 1

## Materials and Methods

### Cells used in Experiment

All experiments were done with electrocompetent NEB10 $\beta$  cells that were mixed in a 1:1 ratio with 30% glycerol solution and frozen at -80° C.

### UP elements

The UP element used in this study, K264006, has been previously characterized by George McArthur, and was the strongest of a library of half a dozen synthetic UP elements designed by the Systems Biological Engineering Lab at VCU<sup>9</sup>.

### Promoter Design and Synthesis

A library of 13 promoters was created for this study, and all but J90001 were based off of the Bba\_J23119 so-called “consensus” sequence from the Anderson promoters library in the MIT Parts Registry (Table 1). In promoters J90002 through J90013 mutations were limited to the -10 hexamer.<sup>10</sup> A subset of these included a -12T->G point mutation due to the deleterious effect of this mutation observed in the Anderson collection. The remaining promoters with the exception of J90010 contained a -11A->G mutation<sup>11</sup>. Positions -10 through -8 were subject to various point transversions. All sequences were surrounded by the standard BioBrick prefix and suffix sequences, as well as additional padding sequences (Table 2). J90001 was synthesized along with UP element K264006 and RFP reporter Bba\_J06702 as an entire UP element/Promoter/Reporter construct. J90002 through J900013 were synthesized *de novo* as ssDNA oligonucleotides by the Nucleic Acids Research Facility (NARF) at VCU and assembled into usable constructs by standard BioBrick assembly.

Promoter	Sequence
BBa_J23119	<b>TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC</b>
J90001	AGGTTCTGTTAAGTAACTGAACCCAATGTCGTTAG
J90002	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GATAATGCTAGC
J90003	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GACAATGCTAGC
J90004	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GATGATGCTAGC
J90005	<b>TTGACAGCTAGCTCAGTCCTAGG</b> TGTAATGCTAGC
J90006	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GACGATGCTAGC
J90007	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GATAGTGCTAGC
J90008	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GATGGTGCTAGC
J90009	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GACAGTGCTAGC
J90010	<b>TTGACAGCTAGCTCAGTCCTAGG</b> TACGGTGCTAGC
J90011	<b>TTGACAGCTAGCTCAGTCCTAGG</b> TGTAGTGCTAGC
J90012	<b>TTGACAGCTAGCTCAGTCCTAGG</b> TGCAATGCTAGC
J90013	<b>TTGACAGCTAGCTCAGTCCTAGG</b> GGTAATGCTAGC

Table 1: Sequences of promoters used in this study. The Bba\_J23119 consensus promoter is listed at the top and was used as the basis for promoters J90002 through J90013. The -35 and -10 hexamer sequences are highlighted in bold, with mutations shown in red. Promoter J90001 is a random sequence not derived from Bba\_J23119.

Component	Sequence
BioBrick Prefix w/ padding	ATCGATCGGAATTCGCGGCCGCTTCTAGAG
BioBrick suffix w/ padding	TACTAGTAGCGGCCGCTGCAGGGAATTCC
Forward-end-padding (Primer)	ATCGATCGGAATTCGCGGCCGCTTCT
Reverse-end-padding JL (Primer)	GGAATTCCTGCAGCGGCC
K264006	GGAAATTTTTTTTTGAAAAGTACT

Table 2: BioBrick padding, PCR primers, and UP element sequences used in this study

### Construct Assembly

#### PCR

Promoter oligos were made double stranded using PCR. Primers (Forward-end-Padding and Reverse-end-padding JL) were designed to anneal to both ends of the promoter oligos and synthesized by NARF. Single stranded oligos from NARF were diluted to concentrations of 100 ng/ $\mu$ L, and 2  $\mu$ L were added to a PCR cocktail consisting of 2.5  $\mu$ L Taq buffer, 16.8  $\mu$ L nanopurified H<sub>2</sub>O, 0.5  $\mu$ L of MgCl<sub>2</sub>, 0.5  $\mu$ L dNTPs, 1.25  $\mu$ L each of both forward and reverse primers (diluted to 20  $\mu$ LM), and 0.2

$\mu\text{L}$  of Taq polymerase. The PCR was run in a BioRad iCycler with the following protocol: 4 min. @  $95^\circ\text{C}$ ; 30 repetitions of 1) 30 sec. @  $95^\circ\text{C}$ , 2) 30 sec. @  $58^\circ\text{C}$ , and 3) 30 sec. @  $72^\circ\text{C}$ ; 10 minutes @  $72^\circ\text{C}$ ;  $4^\circ\text{C}$  hold.

#### *PSB1A3 and PSB1C3 plasmid miniprep*

Isolation of the pSB1A3 (containing J06702) and pSB1C3 (containing the Bba\_P1010 cell death gene) plasmids was performed by inoculating 5 mL of Ampicillin and Chloramphenicol LB, respectively, with scrapings of frozen glycerol stocks of *E. coli* previously transformed with these constructs. Cells were grown at  $37^\circ\text{C}$  overnight (~16 hours) shaking at 175 rpm. 1.5 mL of cells were transferred to microcentrifuge tubes and centrifuged for 4 minutes at 8000 rpm. The pelleted cells were then lysed and the plasmids were isolated using the Qiagen™ Miniprep Kit, following the protocol for spin columns. Plasmids were eluted from the column with 50  $\mu\text{L}$  of nanopurified  $\text{H}_2\text{O}$ .

#### *BioBrick Assembly*

Digestion and Ligation reactions were performed using the NEB BioBrick Assembly Kit (#E0546S). Once promoters were double-stranded, all parts were assembled according to the standard BioBrick assembly protocol with (Fig. 2). 500 ng each of promoter, reporter, and plasmid DNA were each added to PCR tubes for ligation.  $\text{H}_2\text{O}$  was added to the DNA to reach a volume of 42.5  $\mu\text{L}$ . To each tube 5  $\mu\text{L}$  of NEBuffer 2 and 0.5  $\mu\text{L}$  BSA were added. To tubes containing promoters, 1  $\mu\text{L}$  of EcoR1-HF and 1  $\mu\text{L}$  of Spe1 were added. To tubes containing J06702 (pSB1A3), 1  $\mu\text{L}$  Xba1 and 1  $\mu\text{L}$  Pst1 were added. Tubes containing the pSB1C3 target plasmid were treated with 1  $\mu\text{L}$  each of EcoR1-HF and 1  $\mu\text{L}$  Pst1. All tubes were incubated in the thermocycler for 2 hours at  $37^\circ\text{C}$  and then subjected to a 20 minute enzyme denaturation at  $80^\circ\text{C}$ . Digestion products were ligated according to the BioBrick Assembly Protocol. The Spe1 and Xba1 overhangs anneal to each other and form a non-digestible scar region.

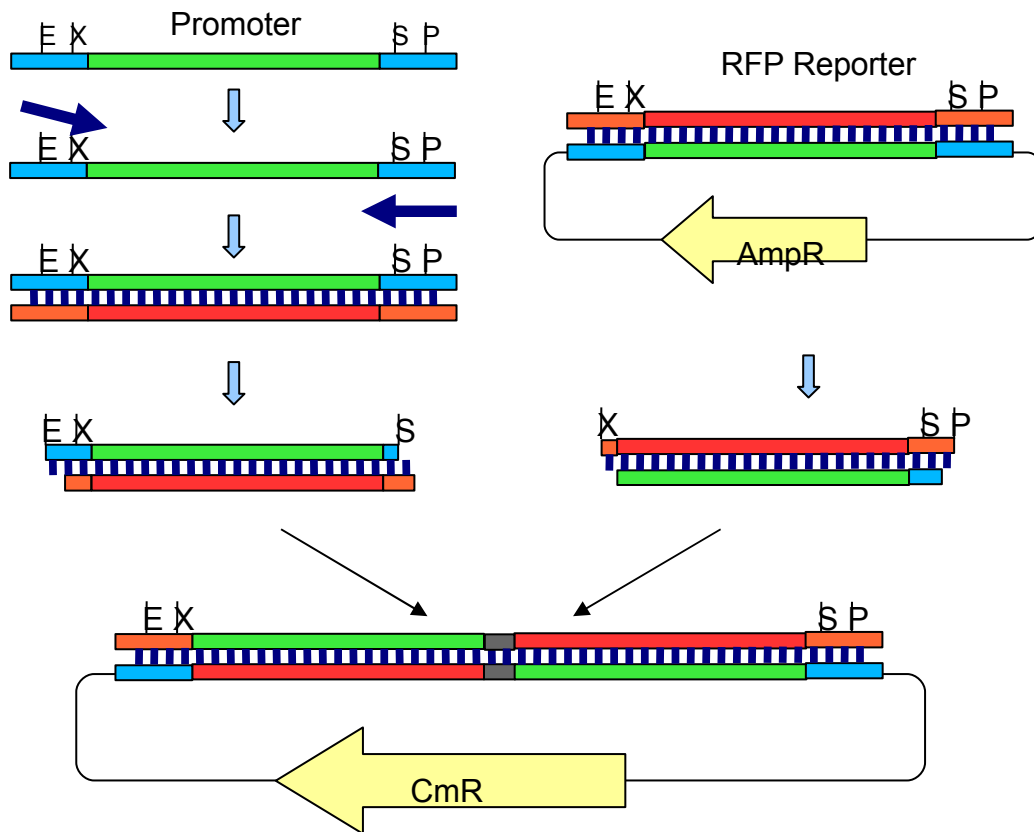


Fig. 2: Schematic of construct assembly. Promoters were first made into dsDNA using PCR, then cut with *EcoRI*-HF and *SpeI*. RFP was cut from the plasmid pSB1A3 using *XbaI* and *PstI*. The two components were ligated into the plasmid pSB1C3, which was cut with *EcoRI*-HF and *PstI*

#### Transformation into *E. coli*

3 $\mu$ L of ligated DNA was added to 50 $\mu$ L of thawed electrocompetent NEB10 $\beta$  *E. coli*. The DNA was mixed with the pipet and allowed to incubate for 1 minute on ice. The cells were then added to BioRad transformation cuvettes, tapped on the counter to get cells to the bottom of the cuvette, and electroporated on setting EC2. 1 mL of SOC was immediately added to the cells and mixed gently. The cells were then incubated for 1 hour at 37 $^{\circ}$  C. They were then spun down for 4 minutes at 8000 rpm. The supernatant was decanted and the cells were resuspended in remaining supernatant (less than 100  $\mu$ L). All cells were then spread on agar plates containing LB and chloramphenicol (CM).

#### Fluorescence Measurement

Three colonies from each construct were picked after 24 hrs and grown individually for 16 hours in tubes containing 5 mL selective LB + CM. The tubes were placed, tilted, in a 37 $^{\circ}$  C incubator shaking at 200 rpm. After the 16 hour incubation, cells were added to 250 mL erlenmeyer flasks containing 15 mL of LB + CM to reach a final OD600 of 0.05 and grown for 3 hours at 37 $^{\circ}$  C, shaking at 175 rpm. The cell line deltaPGI, which doesn't express any fluorescent protein was used as a negative control and underwent all the same procedures, replacing normal LB for LB with CM. After the 3 hours, 0.5 mL of cells were centrifuged for 4 minutes at 8000 rpm and resuspended in 1 mL phosphate-buffered saline. Fluorescence of the resuspended cells was measured using a FACScan flow

cytometer connected to Cellquest Pro software.

## Results

### *Sufficiency of K264006 to initiate transcription*

Strong UP element K264006 was added to the spacer promoter J90001 and placed in front of the coding sequence J06702, which codes for RFP. The fluorescence of the construct was measured and normalized to the fluorescence of the strong promoter J23119. The mean of triplicate measurements gave a relative fluorescence of  $0.13 \pm 0.01$  compared to the J23119 promoter-reporter construct. The negative control also showed 0.13 relative mean fluorescence (not shown). No difference was found between the fluorescence of the K264006-J90001 construct and the negative control (fig 1). The weak promoter J23112 was also measured showed  $0.13 \pm 0.001$  relative fluorescence. Previous fluorescence measurements that were also normalized to the J23119-J06702 construct showed that the addition of UP elements to the weak J23112 promoter restored transcriptional activity.

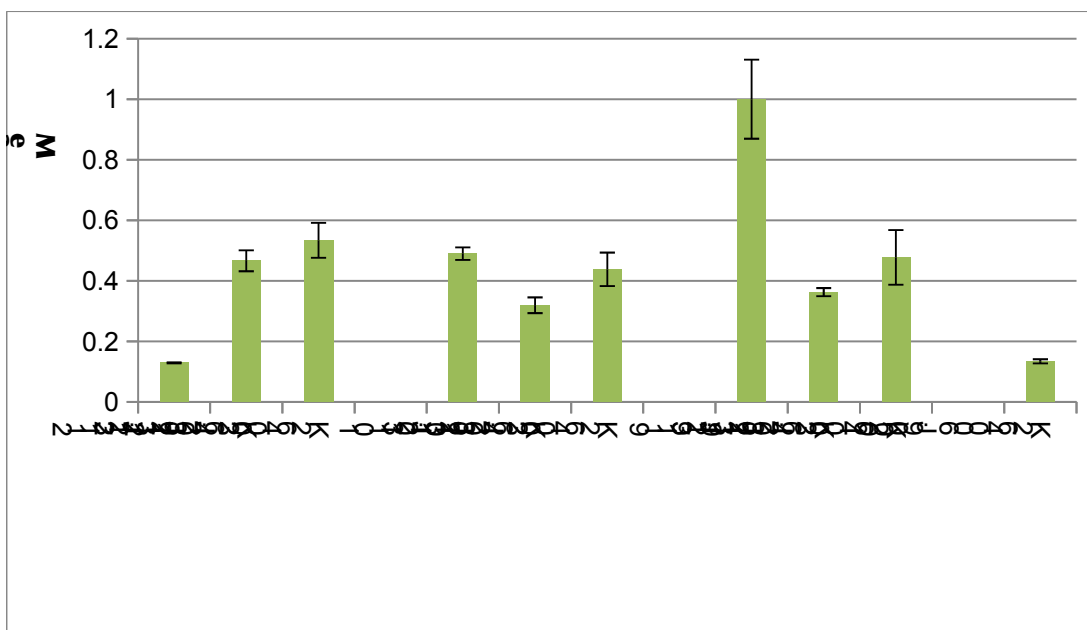


Fig. 3. Relative mean fluorescence of promoters with and without UP elements

### *Characterization of Promoters J90002 through J90013*

The promoters with “sabotaged” -10 hexamer regions were all characterized except for J90007, which failed to yield at least three colonies when transformed into NEB10 $\beta$ . The fluorescence values with standard deviations are provided in Table 3 and Figure 4.

Construct	Mean fluorescence $\pm$ SD
J90002-J06702 (pSB1C3)	3.54 $\pm$ 0.12
J90003-J06702 (pSB1C3)	3.49 $\pm$ 0.25
J90004-J06702 (pSB1C3)	3.8 $\pm$ 0.19
J90005-J06702 (pSB1C3)	3.49 $\pm$ 0.15
J90006-J06702 (pSB1C3)	3.53 $\pm$ 0.04
J90008-J06702 (pSB1C3)	3.44 $\pm$ 0.05
J90009-J06702 (pSB1C3)	3.38 $\pm$ 0.08
J90010-J06702 (pSB1C3)	3.33 $\pm$ 0.10
J90011-J06702 (pSB1C3)	3.50 $\pm$ 0.07
J90012-J06702 (pSB1C3)	3.69 $\pm$ 0.18
J90013-J06702 (pSB1C3)	3.49 $\pm$ 0.17
delta-PGI	3.34 $\pm$ 0.04

Table 3: Mean fluorescence and standard deviations of constructs driving RFP expression

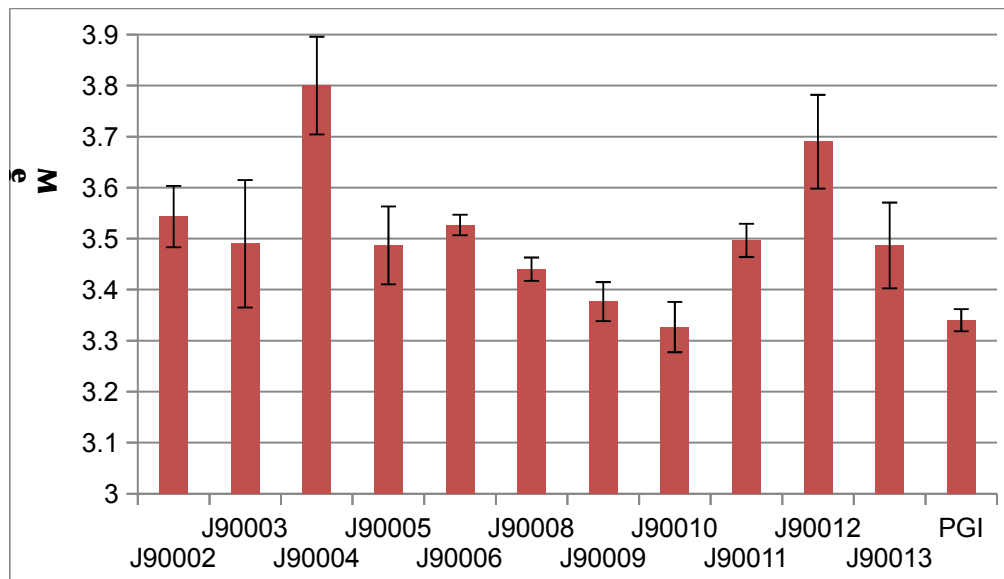


Fig. 4: Mean fluorescence of promoters driving RFP. Error bars denote standard deviation.

## Discussion

The pairing of the strong UP element K264006 with the spacer promoter J90001 showed no detectable level of transcription. From the characterization of the K264006-J90001 construct, we can conclude that this UP element is not sufficient for transcription initiation. This is not surprising, as a large amount of literature has been devoted to underlying the mechanism behind sigma factor's role in recruiting the RNAP holoenzyme<sup>1</sup>. While the UP element has been shown previously to have a strong for the CTD of the RNAP  $\alpha$  subunits<sup>7</sup>, the initiation of transcription is contingent upon the successful

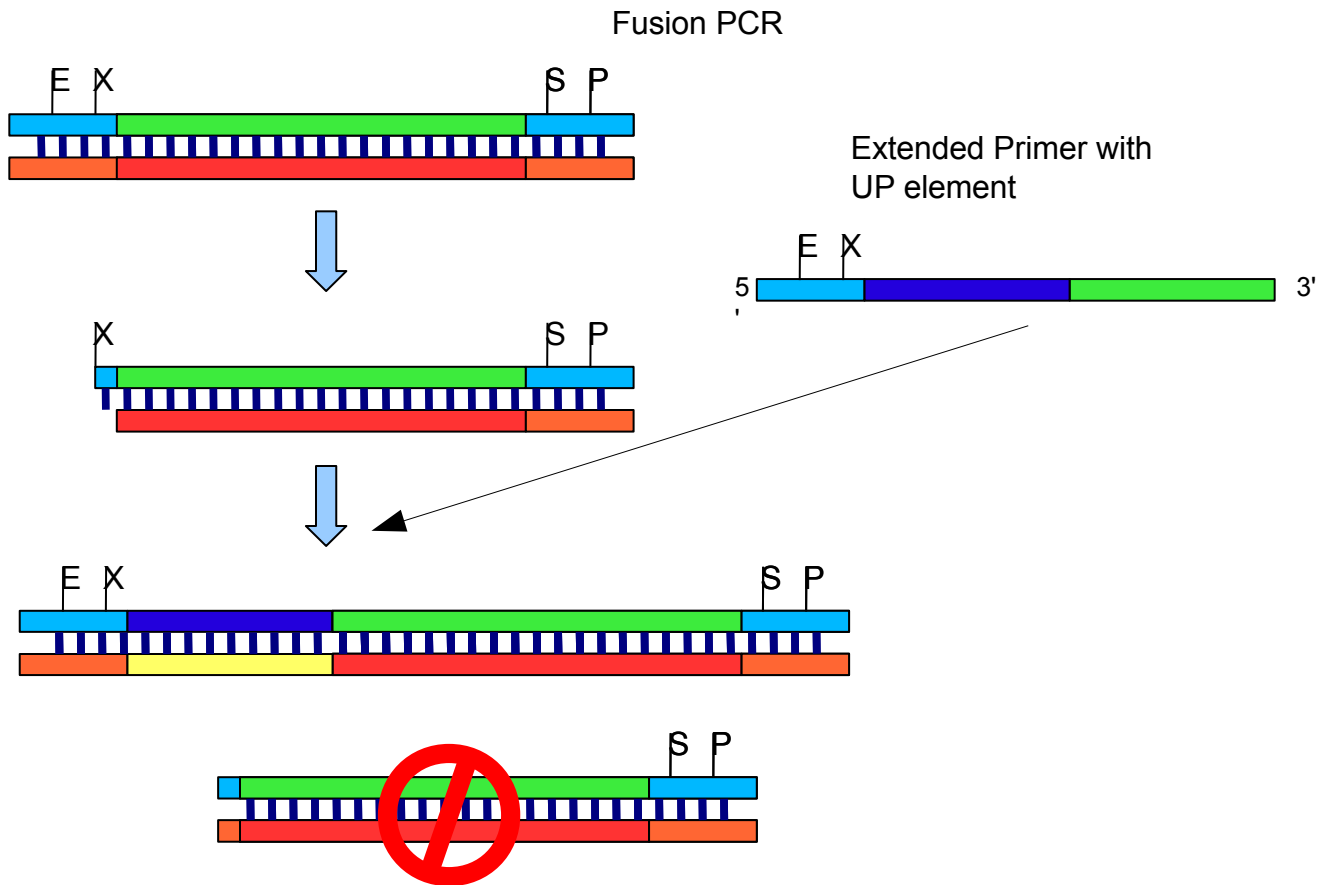
assembly of the RNAP subunits. This ability has not been attributed to the alpha subunits, however. Thus, their affinity for genetic elements is likely not important to the assembly of the prokaryotic transcription machinery.

The promoters used in this study (exclusive of J90001) were designed to test an UP element's ability to restore transcriptional activity to a damaged genetic element. All the promoters showed baseline levels of transcription, but it is unclear whether or not this is due to the inability of sigma factor to bind to the -10 hexamer region or simply an extremely low affinity between the hexamer and protein. To better understand how the binding mechanism has been affected by the mutations, UP elements need to be added to the promoters to see if transcription can take place. This can easily be done *in vitro* with the use of fusion PCR (fig. 5). Additionally, kinetic experiments need to be done to understand exactly how the promoter sequence alters the binding constants to RNAP. This could have useful applications in synthetic biology. For instance, many different genes of interest could be inserted into a cell, but rendered inactive by these weak promoters. UP elements could then serve as the “on/off” switch to activate these genetic pathways, streamlining the process of creating novel pathways *in vivo*.

Our choice of RFP as a reporter mechanism in this study has both positive and negative repercussions. As a visual marker, RFP (as opposed to GFP) can be easily used as a screening device in transformations. However, time lapse microphotography of some of our RFP-expressing constructs show a high variation in RFP expression. Because flow cytometry averages the fluorescence of thousands of individual cells, this source of error should be minimal, but other assays, such as the beta-galactosidase activity assay used in other UP element studies, could yield more consistent results<sup>5,6,7</sup>.

Another important evaluation of transcription levels is qPCR, which measures transcript levels directly, bypassing the problems associated with any particular gene product. The promoters described in this study should be further analyzed with qPCR to confirm the strengths found in this study.

This study aimed at expanding our rather limited knowledge on the applicability of synthetic UP elements to synthetic biological systems. The observation that an UP element can restore expression of a gene with a dysfunctional promoter led us to question our basic model of transcriptional mechanics. The work I completed at BBSI is only a preliminary step in understanding how the synergy of UP elements and promoters (both strong and weak) is responsible for controlling transcriptional regulation at the nucleotide level.



*Fig. 5: Fusion PCR can be used to add UP elements to the promoters using an extended primer that contains the BioBrick prefix, UP element, and a section complimentary to the antisense promoter strand at the 5' end of the promoter. Prior digestion of the promoter with *Xba*I will cause only the PCR product containing the UP element to be usable in BioBrick assembly.*

### Acknowledgements

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