Could you design a genetic code to "fail fast"?

First Position (5′ end)	Second Position: U		Second Position: C		Second Position: A		Second Position: G		Third Position (3′ end)
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	*Stop*	UGA	*Stop*	A
	UUG		UCG		UAG	*Stop*	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

doi:10.1006/jmbi.2000.3658 available online at http://www.idealibrary.com on IDEAL® *J. Mol. Biol.* (2000) **298**, 195–209

JMB

Quadruplet Codons: Implications for Code Expansion and the Specification of Translation Step Size

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**Corresponding author*

One of the requirements for engineering expansion of the genetic code is a unique codon which is available for specifying the new amino acid. The potential of the quadruplet UAGA in *Escherichia coli* to specify a single amino acid residue in the presence of a mutant $tRNA^{Leu}$ molecule containing the extra nucleotide, U, at position 33.5 of its anticodon loop has been examined. With this mRNA-tRNA combination and at least partial inactivation of release factor 1, the UAGA quadruplet specifies a leucine residue with an efficiency of 13 to 26%. The decoding properties of $tRNA^{Leu}$ with U at position 33.5 of its eight-membered anticodon loop, and a counterpart with A at position 33.5, strongly suggest that in both cases their anticodon loop bases stack in alternative conformations. The identity of the codon immediately 5′ of the UAGA quadruplet influences the efficiency of quadruplet translation *via* the properties of its cognate tRNA. When there is the potential for the anticodon of this tRNA to dissociate from pairing with its codon and to re-pair to mRNA at a nearby 3′ closely matched codon, the efficiency of quadruplet translation at UAGA is reduced. Evidence is presented which suggests that when there is a purine base at position 32 of this 5′ flanking tRNA, it influences decoding of the UAGA quadruplet.

Keywords: quadruplet codon; code expansion; frameshift suppressors; anticodon; frameshifting

Biotechnol. Prog. **1999,** *15,* 58–64

Library of Synthetic 5′ Secondary Structures To Manipulate mRNA Stability in *Escherichia coli*

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A DNA cassette system has been developed to allow for the convenient introduction of synthetic DNA oligonucleotides between the transcription and translation start sites of a gene in order to examine the effect of 5′ hairpin structure and strength on mRNA stabilization. Rationally designed synthetic DNA cassettes were introduced into the 5′ untranslated region of a modified *lacZ* gene to form hairpins at the 5′ end of the mRNA. These DNA inserts influenced mRNA half-lives over an order-of-magnitude range, with some groups of predicted structures having half-lives that showed a strong correlation with hairpin strength while half-lives for another group of predicted structures exhibited little or no dependence on this property. These results indicate the importance of 5′ hairpin structure and strength in determining stabilization of *Escherichia coli* mRNA. This synthetic library, as well others generated using the DNA cassette system described here, should prove useful in understanding the mechanisms of mRNA stabilization and in designing structures for recombinant gene expression control.

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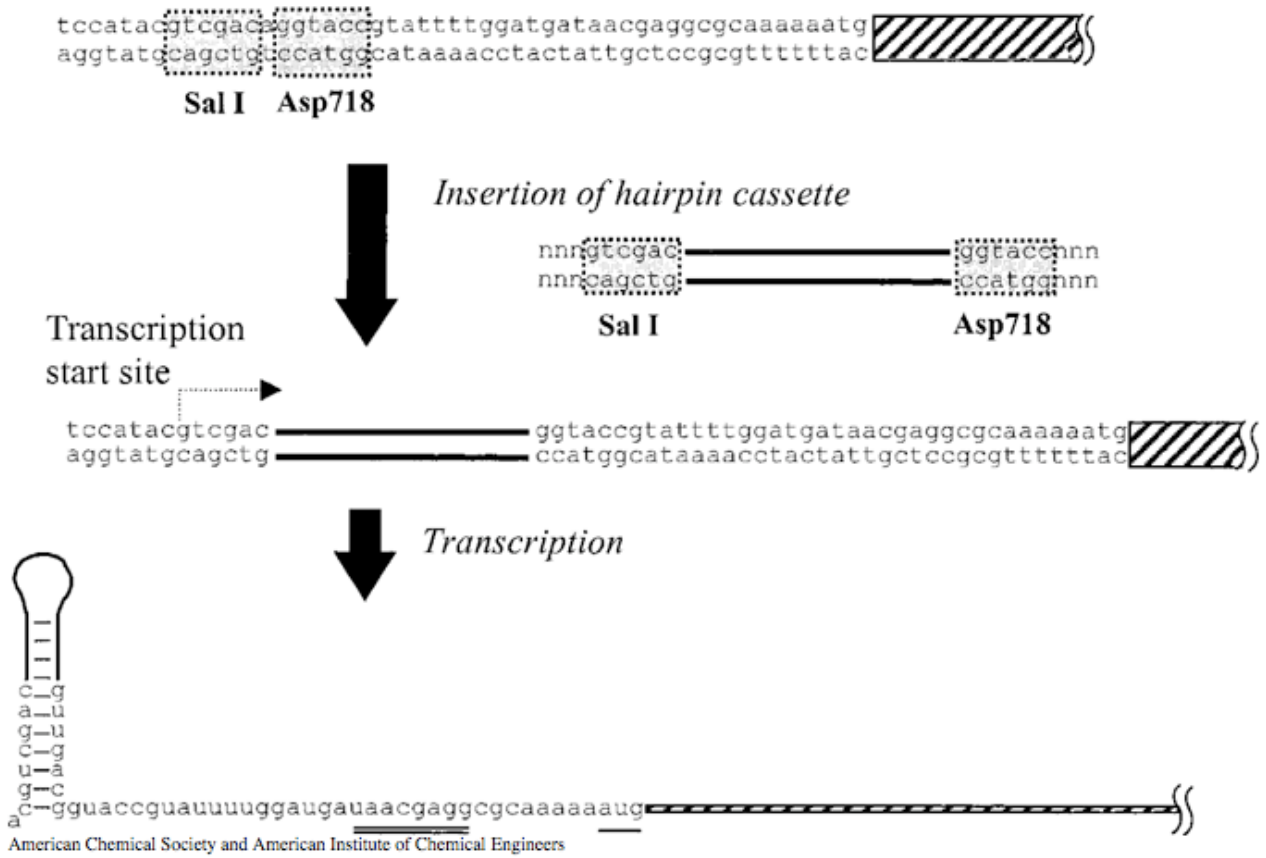
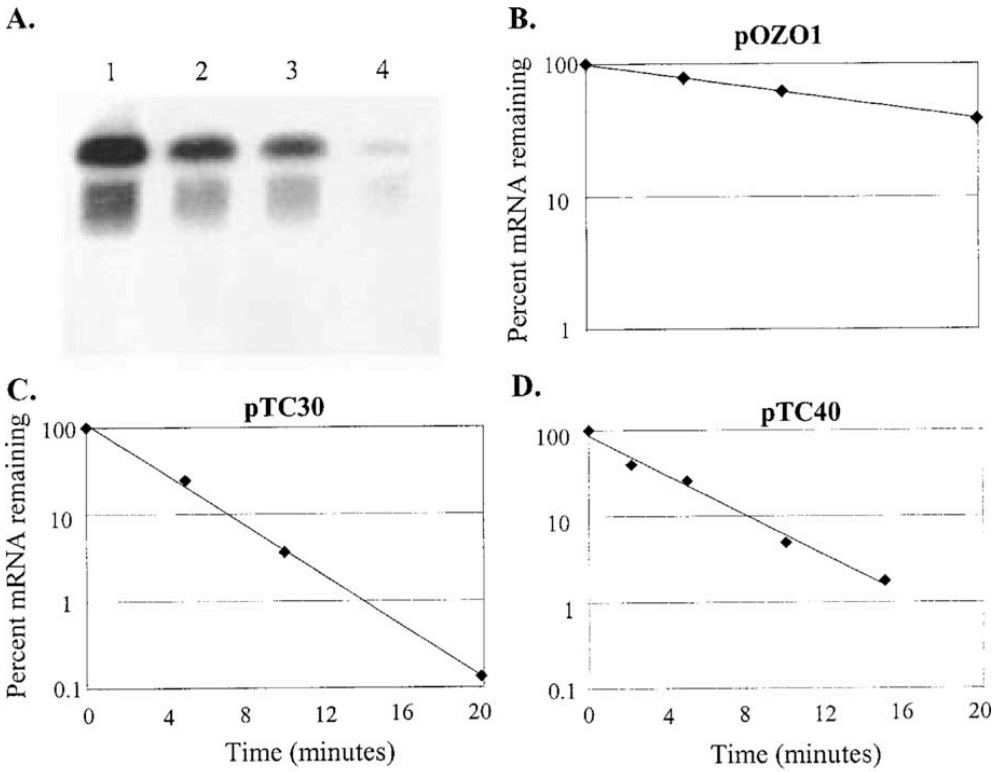


Figure Figure 1.. Strategy for inserting synthetic DNA cassettes into the 5′ untranslated region of a gene. Double underlining indicates the Shine-Delgarno region, and single underlining indicates translation start codon.


A.
1
2
3
4
B.
pOZO1
Percent mRNA remaining
100
10
1
C.
pTC30
Percent mRNA remaining
100
10
1
0.1
0
4
8
12
16
20
Time (minutes)
D.
pTC40
100
10
1
0.1
0
4
8
12
16
20
Time (minutes)
E.
pOZO1
gau aaggauuuaa
t1/2 = 14.7 min (+/- 0.1)
pTC30
acgucga ggauuuaa
t1/2 = 2.1 min (+/- 0.1)
pTC40
acgucgacagguaccguauuuu
t1/2 = 2.6 min (+/- 0.2)

pHP4

$t_{1/2}$ = 8.3 min (+/- 0.5)
$\Delta G_{folding}$ = -16.5 kcal/mol

pHP5

$t_{1/2}$ = 5.6 min (+/- 0.4)
$\Delta G_{folding}$ = -26.2 kcal/mol

pHP8

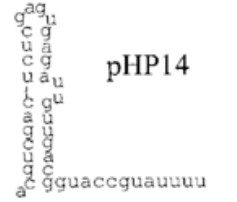
$t_{1/2}$ = 6.1 min (+/- 0.2)
$\Delta G_{folding}$ = -18.0 kcal/mol

pHP9

$t_{1/2}$ = 5.5 min (+/- 0.1)
$\Delta G_{folding}$ = -20.8 kcal/mol

pHP10

$t_{1/2}$ = 6.8 min (+/- 0.4)
$\Delta G_{folding}$ = -21.8 kcal/mol



pHP14

$t_{1/2}$ = 4.9 min (+/- 0.2)
$\Delta G_{folding}$ = -13.7 kcal/mol

pHP15

$t_{1/2}$ = 2.1 min (+/- 0.2)
$\Delta G_{folding}$ = -9.5 kcal/mol

pHP16

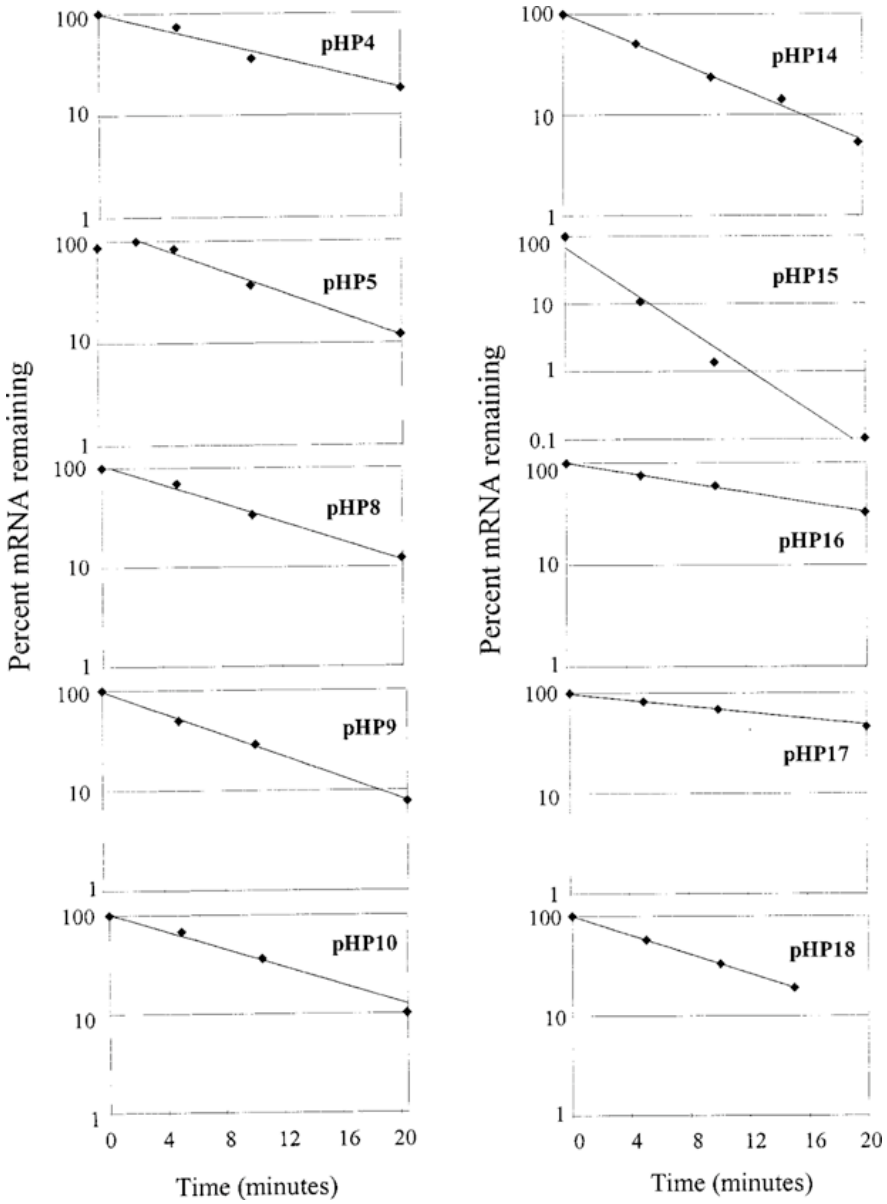
$t_{1/2}$ = 12.5 min (+/- 0.4)
$\Delta G_{folding}$ = -25.0 kcal/mol

pHP17

$t_{1/2}$ = 19.8 min (+/- 0.9)
$\Delta G_{folding}$ = -15.9 kcal/mol

pHP18

$t_{1/2}$ = 6.3 min (+/- 0.0)
$\Delta G_{folding}$ = -15.7 kcal/mol


pHP4
pHP5
pHP8
pHP9
pHP10
pHP14
pHP15
pHP16
pHP17
pHP18
Percent mRNA remaining
Time (minutes)
100
10
1
0.1
0
4
8
12
16
20

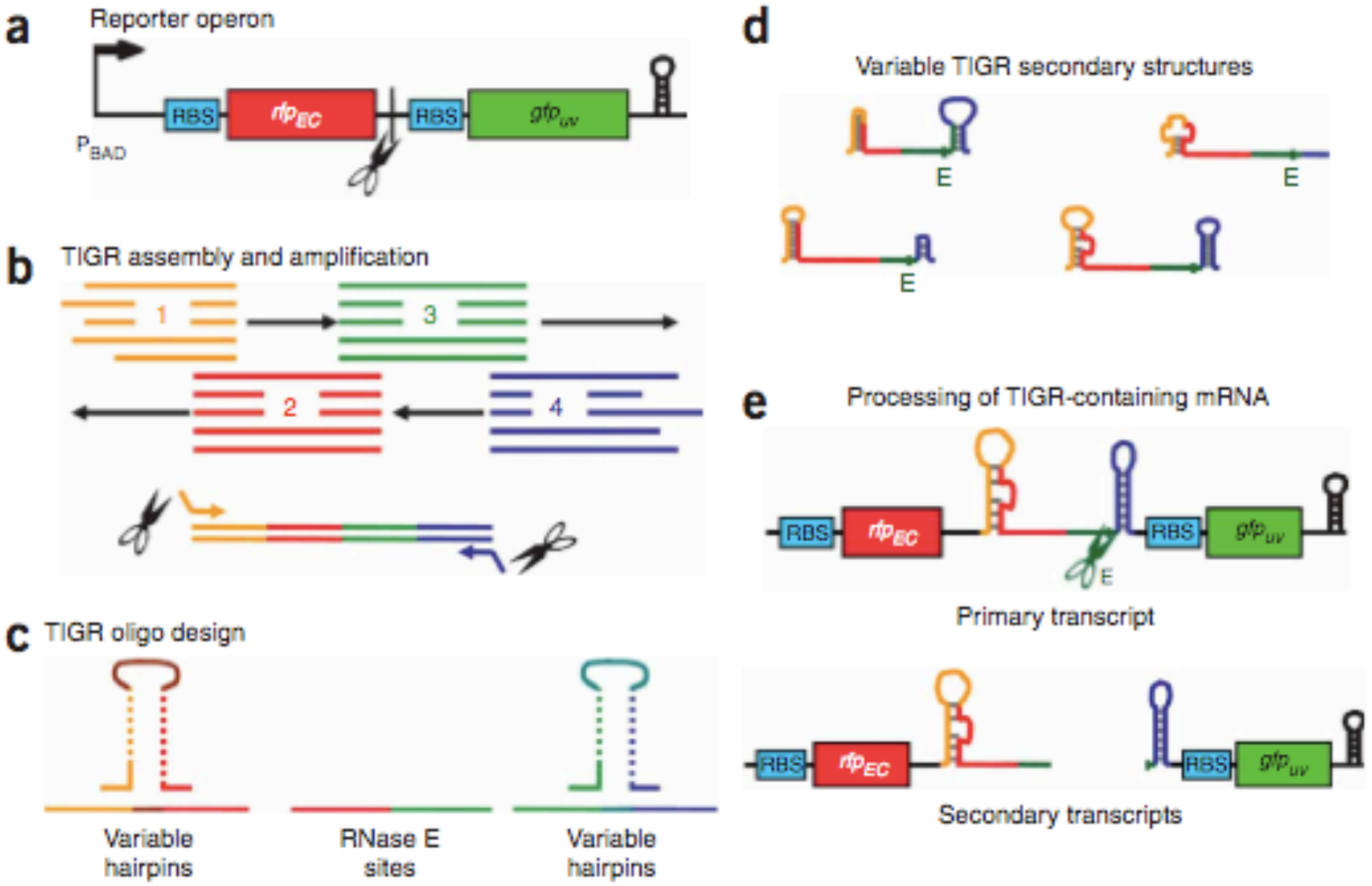
Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes

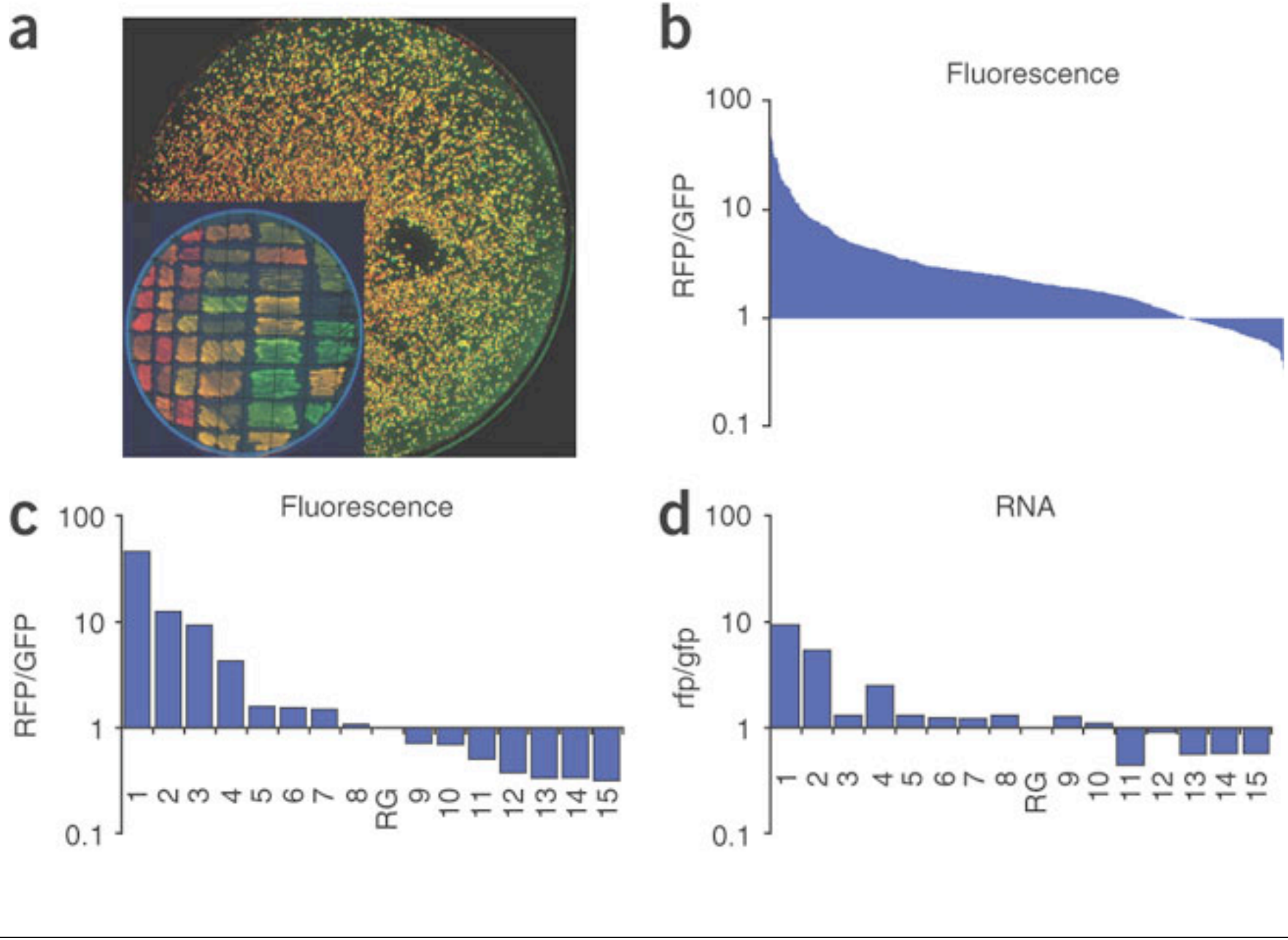
Brian F Pfleger[1], Douglas J Pitera[1], Christina D Smolke[1,4], & Jay D Keasling[1–3]

Many applications of synthetic biology require the balanced expression of multiple genes. Although operons facilitate coordinated expression of multiple genes in prokaryotes and eukaryotes, coordinating the many post-transcriptional processes that determine the relative levels of gene expression in operons by *a priori* design remains a challenge. We describe a method for tuning the expression of multiple genes within operons by generating libraries of tunable intergenic regions (TIGRs), recombining various post-transcriptional control elements and screening for the desired relative expression levels. TIGRs can vary the relative expression of two reporter genes over a 100-fold range and balance expression of three genes in an operon that encodes a heterologous mevalonate biosynthetic pathway, resulting in a sevenfold increase in mevalonate production. This technology should be useful for optimizing the expression of multiple genes in synthetic operons, both in prokaryotes and eukaryotes.

eukaryotes[6–9]. With a single promoter controlling the transcription of several genes, relative expression of each open reading frame in the operon is controlled by altering post-transcriptional processes such as transcription termination[10,11], mRNA stability[12,13] and translation initiation[14–16]. Sequences inserted into the intergenic regions of bacterial operons can direct the processing and segmental stability of a transcript containing multiple coding regions[17,18]. This type of directed mRNA processing results in differential production of the proteins encoded in the operon depending on the nature of the intergenic region between the coding regions.

One of the major obstacles to designing and implementing this type of control is the difficulty in decoupling the many interrelated variables involved in post-transcriptional regulation[19]. We previously demonstrated that it is possible to differentially control the protein levels encoded by two or more genes in an operon using intergenic-region sequences[17,18]. Here, we simultaneously tune the expression of several genes within operons by generating and screening large


a
Reporter operon
RBS
rfp_EC
RBS
gfp_uv
P_BAD
b
TIGR assembly and amplification
1
2
3
4
c
TIGR oligo design
Variable hairpins
RNase E sites
Variable hairpins
d
Variable TIGR secondary structures
E
E
E
e
Processing of TIGR-containing mRNA
RBS
rfp_EC
RBS
gfp_uv
E
Primary transcript
RBS
rfp_EC
RBS
gfp_uv
Secondary transcripts


a
b
Fluorescence
100
10
1
0.1
RFP/GFP
c
Fluorescence
100
10
1
0.1
RFP/GFP
1
2
3
4
5
6
7
8
RG
9
10
11
12
13
14
15
d
RNA
100
10
1
0.1
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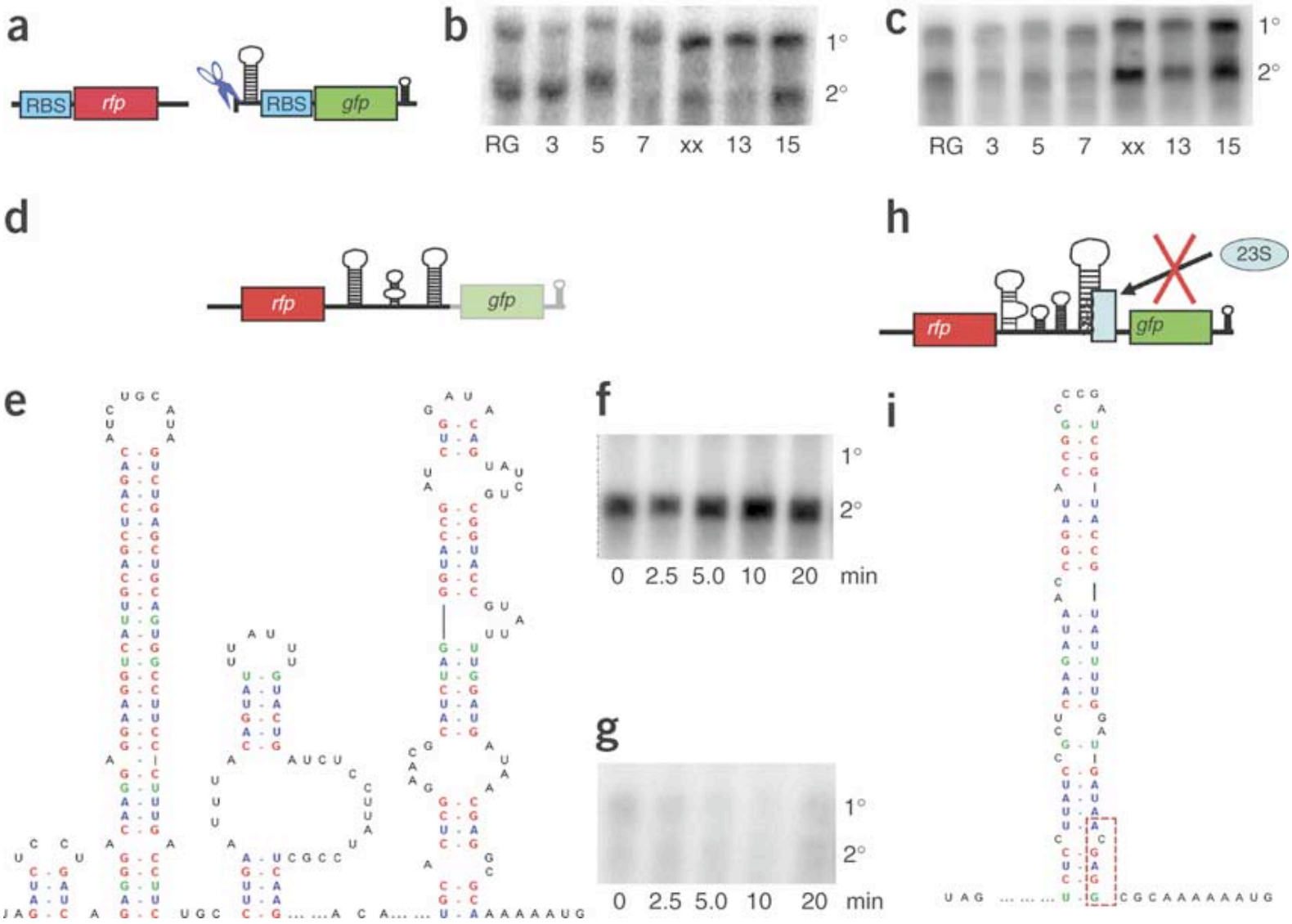
1
2
3
4
5
6
7
8
RG
9
10
11
12
13
14
15


a
RBS
rfp
RBS
gfp
b
1°
2°
RG 3 5 7 xx 13 15
c
1°
2°
RG 3 5 7 xx 13 15
d
rfp
gfp
h
23S
rfp
gfp
e
f
1°
2°
0 2.5 5.0 10 20 min
g
1°
2°
0 2.5 5.0 10 20 min
i

A modular and extensible RNA-based gene-regulatory platform for engineering cellular function

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Edited by Arthur D. Riggs, Beckman Research Institute, City of Hope, Duarte, CA, and approved July 12, 2007 (received for review May 1, 2007)

Engineered biological systems hold promise in addressing pressing human needs in chemical processing, energy production, materials construction, and maintenance and enhancement of human health and the environment. However, significant advancements in our ability to engineer biological systems have been limited by the foundational tools available for reporting on, responding to, and controlling intracellular components in living systems. Portable and scalable platforms are needed for the reliable construction of such communication and control systems across diverse organisms. We report an extensible RNA-based framework for engineering ligand-controlled gene-regulatory systems, called ribozyme switches, that exhibits tunable regulation, design modularity, and target specificity. These switch platforms contain a sensor domain, comprised of an aptamer sequence, and an actuator domain, comprised of a hammerhead ribozyme sequence. We examined two modes of standardized information transmission between these domains and demonstrate a mechanism that allows for the reliable and modular assembly of functioning synthetic RNA switches and regulation of ribozyme activity in response to various effectors. In addition to demonstrating examples of small

lack portability across organisms and systems, and their designs and construction do not support modularity and component reuse.

We set out to develop a universal and extensible RNA-based platform that will provide a framework for the reliable design and construction of gene-regulatory systems that can control the expression of specific target genes in response to various effector molecules.[†] We implemented five engineering design principles (DPs) in addressing this challenge: DP1, scalability (a sensing platform enabling *de novo* generation of ligand-binding elements for implementation within the sensor domain); DP2, portability (a regulatory element that is independent of cell-specific machinery or regulatory mechanisms for implementation within the actuator domain); DP3, utility (a mechanism through which to modularly couple the control system to functional level components); DP4, composability (a mechanism by which to modularly couple the actuator and sensor domains without disrupting the activities of these individual elements); and DP5, reliability (a mechanism through which to standardize the transmission of information from the sensor domain to the actuator domain).

Results

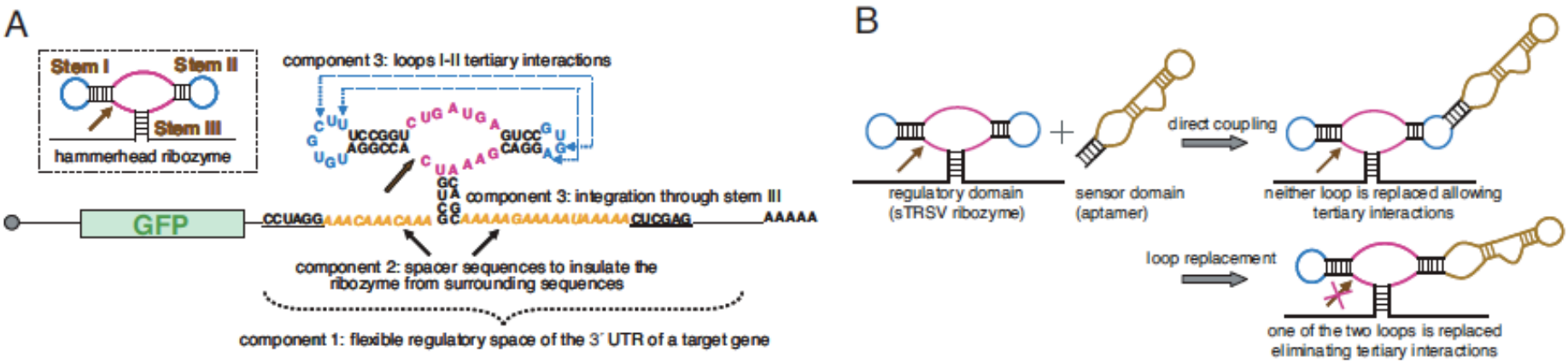

Fig. 1. General design strategy for engineering ribozyme switches. The color scheme is as follows: catalytic core, purple; aptamer sequences, brown; loop sequences, blue; spacer sequences, yellow; brown arrow, cleavage site. (*A*) General compositional framework and design strategy for engineering cis-acting hammerhead ribozyme-based regulatory systems. Restriction enzyme sites are underlined. (*B*) Modular coupling strategies of the sensor and regulatory domains to maintain *in vivo* activity of the individual domains.

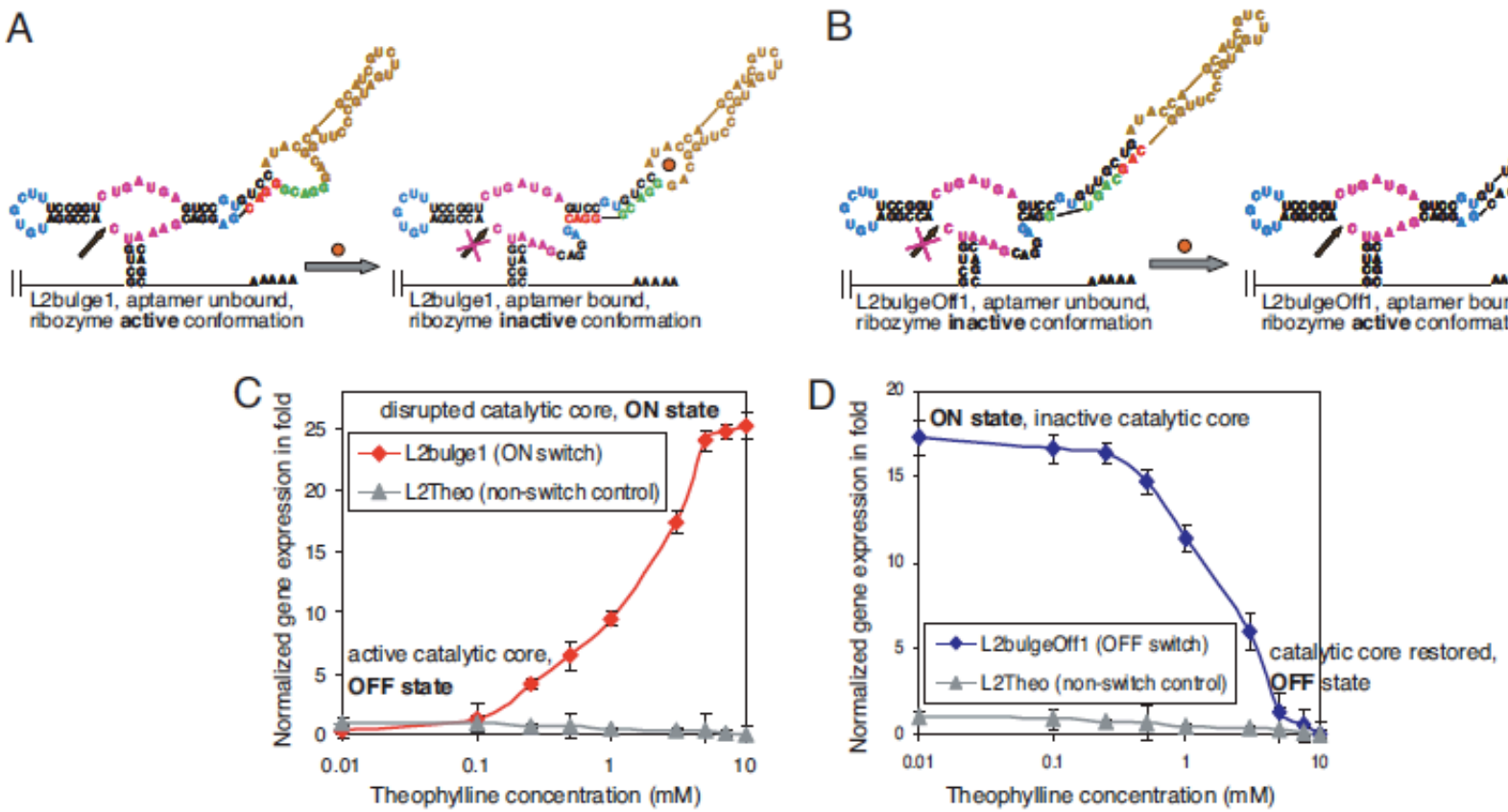

Fig. 2. Regulatory properties of the strand-displacement information transmission mechanism. The color scheme corresponds to that used in Fig. 1 with the following exceptions: switching strand, red; competing strand, green. (*A*) Gene expression ON ribozyme switch platform, L2bulge1. (*B*) Gene expression OFF ribozyme switch platform, L2bulgeOff1. (*C* and *D*) The theophylline-dependent gene-regulatory behavior of L2bulge1 (ON switch) (*C*), L2bulgeOff1 (OFF switch) (*D*), and L2Theo (nonswitch control). Gene-expression levels are reported in fold as defined in *SI Text* and were normalized to the expression levels in the absence of effector.