A light-switchable gene promoter system

Sae Shimizu-Sato, Enamul Huq, James M. Tepperman, and Peter H. Quail*

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Regulatable transgene systems providing easily controlled, conditional induction or repression of expression are indispensable tools in biomedical and agricultural research and biotechnology. Several such systems have been developed for eukaryotes[1–6]. Most of these rely on the administration of either exogenous chemicals or heat shock. Despite the general success of many of these systems, the potential for problems, such as toxic, unintended, or pleiotropic effects of the inducing chemical or treatment, can impose limitations on their use. We have developed a promoter system that can be induced, rapidly and reversibly, by short pulses of light. This system is based on the known red light–induced binding of the plant photoreceptor phytochrome to the protein PIF3 and the reversal of this binding by far-red light[7,8]. We show here that yeast cells expressing two chimeric proteins, a phytochrome–GAL4-DNA-binding-domain fusion and a PIF3–GAL4-activation-domain fusion, are induced by red light to express selectable or "scorable" marker genes containing promoters with a GAL4 DNA-binding site, and that this induction is rapidly abrogated by subsequent far-red light. We further show that the extent of induction can be controlled precisely by titration of the number of photons delivered to the cells by the light pulse. Thus, this system has the potential to provide rapid, noninvasive, switchable control of the expression of a desired gene to a preselected level in any suitable cell by simple exposure to a light signal.

The phytochromes are a family of sensory photoreceptors (designated phyA through phyE) that regulate plant growth and development in response to informational light signals[9]. These molecules are chromoproteins comprising a polypeptide and a covalently linked tetrapyrrole chromophore. The polypeptide folds into two main domains: the photoactive N-terminal domain that contains the chromophore, and the non-chromophoric C-terminal domain[10]. The holoprotein has two forms that are reversibly interconvertible by light: the biologically inactive Pr form that absorbs red light, and the biologically active Pfr form that absorbs far-red light[9]. When the Pr form absorbs a photon, it is converted to the Pfr form, and when the Pfr form absorbs a photon, it is converted back to the Pr form. This photo-interconversion process is complete within milliseconds for any given molecule and is indefinitely repeatable. The molecule is synthesized in the Pr form. Thus, for cells maintained in darkness, the photoreceptor is accumulated in this inactive form, poised for activation on exposure to light.

PIF3, a basic helix–loop–helix protein, interacts selectively with the Pfr form of both phyA and phyB *in vitro* upon light activation[7,8]. We have exploited this light-dependent, conformer-specific interaction to develop a light-switchable gene expression system. The general design of this system, which is based on the yeast two-hybrid concept[11], is described in Figure 1A.

In initial feasibility tests, we examined phyA and phyB constructs in both selectable and scorable two-hybrid assay conditions. Both full-length phyA and the photosensory N-terminal domain of phyB, each fused to the GAL4 DNA-binding domain (phyA(FL)–GBD and phyB(NT)–GBD, respectively), supported colony growth exclusively in red light on selection medium containing the chromophore phycocyanobilin (PCB) when co-transformed with the PIF3–GAL4-activation-domain fusion protein (PIF3–GAD; Fig. 1B). Red light was ineffective in the absence of exogenous chromophore, and the chromophore was ineffective in darkness. We confirmed and quantified these results using LacZ marker expression in non-selective, liquid-culture assay conditions (Fig. 1C). This system can theoretically function in any cell or organism that can either synthesize the chromophore (PCB) endogenously or take up exogenously supplied PCB. A cell or organism might also be genetically engineered to synthesize PCB and assemble holophytochrome as shown in bacteria[12].

We selected the phyB(NT)–GBD/PIF3–GAD combination of constructs to optimize the system. To maximize inducible expression, it is desirable to have maximal levels of the fully assembled photoreceptor fusion protein in the cell poised to respond to the light signal. This requires synthesis of the protein, import of the chromophore, and assembly of the photoactive molecule. We therefore examined the effects of various preincubation conditions on photoinducibility. Cells preincubated with the chromophore in darkness, and subsequently during irradiation, showed the highest levels of induction (Fig. 2A). We therefore adopted this step as standard procedure.

LacZ inducibility is maximized by a pulse of red light of only 1 min followed by further incubation in the dark (Fig. 2B). Continuous light of 30 min or more reduced activity, with strong reduction after 3 h of light (note scale differences in Fig. 2A, B), possibly due to photodynamic damage induced by excess free chromophore. These data indicate that a single short pulse of light that maximizes conversion of preexisting phyB molecules to the active Pfr form at the start of the culture period maximizes induction during the subsequent dark period.

The photochemical properties of the phytochrome molecule make it possible to accurately control the number of molecules converted to the Pfr form by controlling the number of red photons delivered in a pulse irradiation. The fluence (dose)–response curve for LacZ induction by a single red-light pulse is shown in Figure 2C. This curve closely follows well-established data for the phytochromes[13] and indicates the photochemical and functional integrity of the photoreceptor in the yeast cells. The data demonstrate that this system can be used to provide a precisely regulated and reproducible level of expression of the target gene by delivering a predefined number of photons in a single, short light pulse at the start of the culture period.

The unique photoreversibility of the phytochrome system makes it possible to use the photoreceptor as a molecular switch. The red light–induced binding of phyB to PIF3 *in vitro* is rapidly reversed by subsequent far-red light[7] (Fig. 1A). To determine whether this property could be used to reverse the induction of LacZ activity in our system in cells, pulse-red-light-induced cells were subsequently exposed to pulses of far-red light immediately or at 0.5, 1, or 2 h after the start of the culture period (Fig. 2D). The data show that the far-red pulse blocked any further apparent increase in activity beyond that reached at the time of this pulse. It seems, therefore, that reconversion of the Pfr form to the Pr form in the yeast cell effectively terminates the red-light-induced expression, presumably by dissociation of PIF3–GAD from the photoreceptor.

To determine more accurately how rapidly expression can be induced and repressed in our system, we did shorter-term time-course analysis (Fig. 2E). The data show that LacZ activity was induced 2-fold by 5 min, 4-fold by 10 min, and 50-fold by 30 min after a 1-min saturating red-light pulse. Conversely, a decrease in the rate of LacZ accumulation was detectable within 10 min of a far-red-light pulse given to fully induced cells 30 min after the initial red

Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, and US Department of Agriculture, Agricultural Research Service, Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710.
**Corresponding author (quail@nature.berkeley.edu).*

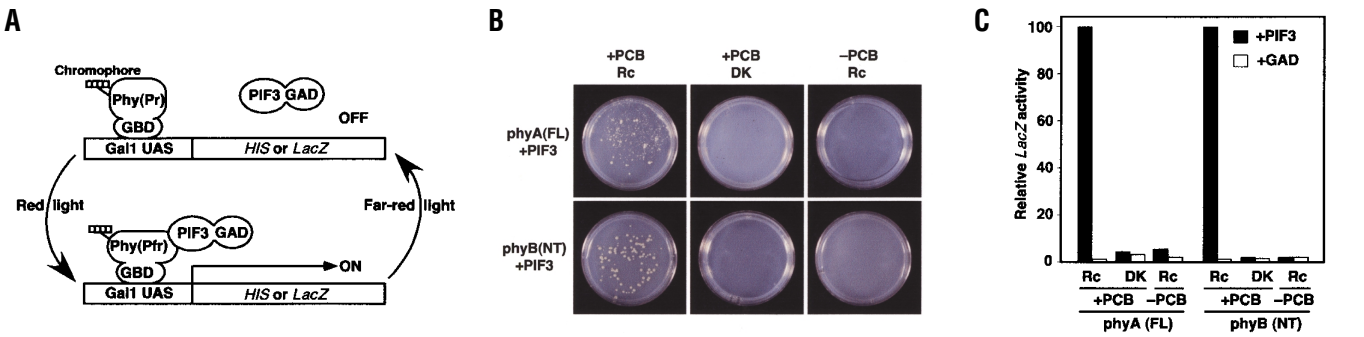

Figure 1. Light-responsive gene promoter system. (A) Transformed cells are maintained in darkness until target gene expression is activated by light. In darkness, the phy(Pr)–GBD fusion protein is synthesized, and the chromophore is attached to the phytochrome moiety to generate the biologically inactive Pr conformer. This fusion protein will bind to its DNA-binding site in the target gene promoter through its GAL4 DNA-binding domain, but because the phytochrome moiety is in the Pr conformer, it will not bind to the PIF3–GAD protein. In this configuration, the target gene is off. To activate expression of the target gene, cells are exposed to red light. This light wavelength converts the phytochrome moiety within 1 s to the biologically active Pfr conformer of the photoreceptor that binds the PIF3 moiety of the PIF3–GAD fusion protein with high affinity. The photoactivated phy(Pfr)–GBD molecule thereby recruits the PIF3–GAD protein to the target promoter, where the transcriptional activation domain of PIF3–GAD induces transcription of the target gene. To switch off expression of this gene, cells are exposed to far-red light. This wavelength of light switches the phytochrome molecule back to the inactive Pr conformer within 1 s, causing rapid dissociation of PIF3–GAD and termination of the transcriptional activation of the target gene. This overall activation–deactivation process can be repeated as needed using sequential exposure of cells to red and far-red light. (B, C) Photoactive phyA and phyB recombinant proteins interact specifically in the Pfr form with PIF3 in living yeast cells. (B) Yeast two-hybrid plate-growth assay. Yeast cells (AH109) were transformed with plasmids encoding a full-length phyA (phyA(FL))–GBD fusion protein and a PIF3–GAD fusion protein (phyA(FL) + PIF3), or phyB N-terminal domain (phyB(NT))–GBD fusion protein and the PIF3–GAD fusion protein (phyB(NT) + PIF3). The transformants were cultured on selective media in the presence (+) or absence (–) of 25 μM PCB under continuous red light (Rc) or in darkness (DK) for 3 days. The yeast cells grew only on the selective media containing PCB under continuous red light. (C) Quantitative yeast two-hybrid liquid assay. Yeast cells (Y187) were transformed (horizontal axis, constructs) and cultured in the presence (+) or absence (–) of 25 μM PCB under continuous red light (Rc) or in darkness (DK). LacZ activity was measured and is expressed in relative units. Black bars, relative LacZ activities for phy–GBD with PIF3–GAD; white bars, relative LacZ activities for phy–GBD with GAD only. Phytochromes–GBD and PIF3–GAD show strong induction of LacZ activities in the presence of PCB in continuous red light.

pulse. Cessation of further LacZ accumulation was complete by 15 min after the far-red pulse. The speed of light-triggered induction and cessation of LacZ accumulation appears to be more rapid than that reported for galactose induction in yeast[14].

The absolute levels of expression provided by our optimized system compared with that of the native GAL4 protein under the same conditions are shown in Table 1. The expression level for the phyB–PIF3 pair in darkness was indistinguishable from the background level of the GBD–GAD negative control. A single red-light pulse induced expression 1,000-fold or more above background, and a subsequent far-red pulse completely blocked induction above this background level. In absolute terms, the level of red light–induced expression was about one-sixth that of the native GAL4 protein in these conditions (Table 1). These data show that although the absolute expression level of the light-inducible two-component system is less than that of the intact, parent GAL4 activator, expression is nevertheless inducible over a large dynamic range above a background level that is negligible in the absence of the inducer, and that induction is fully abrogated by switching off of the activated state of the phyB molecule.

Table 1. Basal and light-inducible expression levels compared with native GAL4 activation

Introduced constructs	LacZ activity (MU)		
	Dark	Rp	Rp–FRp
GBD + GAD	0.1 ± 0.03	0.2 ± 0.03	0.2 ± 0.05
phyB(NT)–GBD + GAD	0.2 ± 0.05	0.1 ± 0.04	0.1 ± 0.03
phyB(NT)–GBD + PIF3–GAD	0.1 ± 0.03	191 ± 2	0.1 ± 0.03
GBD–GAD (native GAL4 protein)	1,146 ± 9	1,127 ± 10	1,138 ± 5

Yeast cells were transformed, cultured, and assayed as in Figure 2C. Transformed cells were either kept in the dark, exposed to a single red-light pulse (12×10^3 μmol m^{-2}) and returned to darkness for 3 h (Rp), or exposed to the Rp followed immediately by a far-red-light pulse (18.6×10^3 μmol m^{-2}) and returned to darkness for 3 h (Rp–FRp), and then assayed for LacZ reporter activity. GBD, GAL4 DNA-binding domain; GAD, GAL4 activation domain; phyB(NT)–GBD, phyB(NT)–GBD fusion protein; PIF3–GAD, PIF3–GAD fusion protein; GBD–GAD, native GAL4 protein. LacZ assays were done in triplicate and the data represent mean ± standard error. M.U., Miller units.

Various authors have outlined the features of the ideal regulatable gene expression system[3–5]. The light-switchable system described here has most, if not all, of these features. Basal levels of expression are low, but rapidly and reversibly induced to high levels by the light signal (Fig. 2; Table 1). As the interacting factors are poised in the nucleus and red light–induced Pr to Pfr conversion occurs within milliseconds, transcriptional induction can potentially initiate within seconds. Reversal of induction may be similarly rapid, requiring only dissociation of PIF3 from phyB after far-red-light-induced Pfr-to-Pr conversion[7]. This property permits highly synchronous and uniform induction across cell populations. The level of expression is precisely controllable (Fig. 2C). This is because the extent of photoconversion of Pr to Pfr is directly proportional to the number of red photons delivered in a highly predictable and reproducible manner[13]. In addition to providing this precise dose control, light is an inexpensive, universally available, simple-to-use, nontoxic inducer that is readily usable with many systems. Any cells to which light can be delivered are potential targets, including single-celled organisms, cultured cells, and those in light-penetrant multicellular organisms such as worms, flies, and plants. Directed light delivery by fiber optics has the potential to target selected cells or tissues, even within larger, more-opaque organisms, thus providing the opportunity to investigate the consequences of cell-specific expression and possible therapeutic applications. Although the present configuration of the light-switchable system provides inducible gene expression, the polarity may be reversed to provide reversible light-imposed repression of expression. It is anticipated that exogenously supplied chromophore, as used here, will be readily absorbed by other cell systems, including multicellular organisms, such as flies and worms, given that *Arabidopsis thaliana* mutants null for chromophore biosynthesis are fully rescued by PCB exogenously supplied to whole seedlings[15]. Alternatively, it is possible to engineer

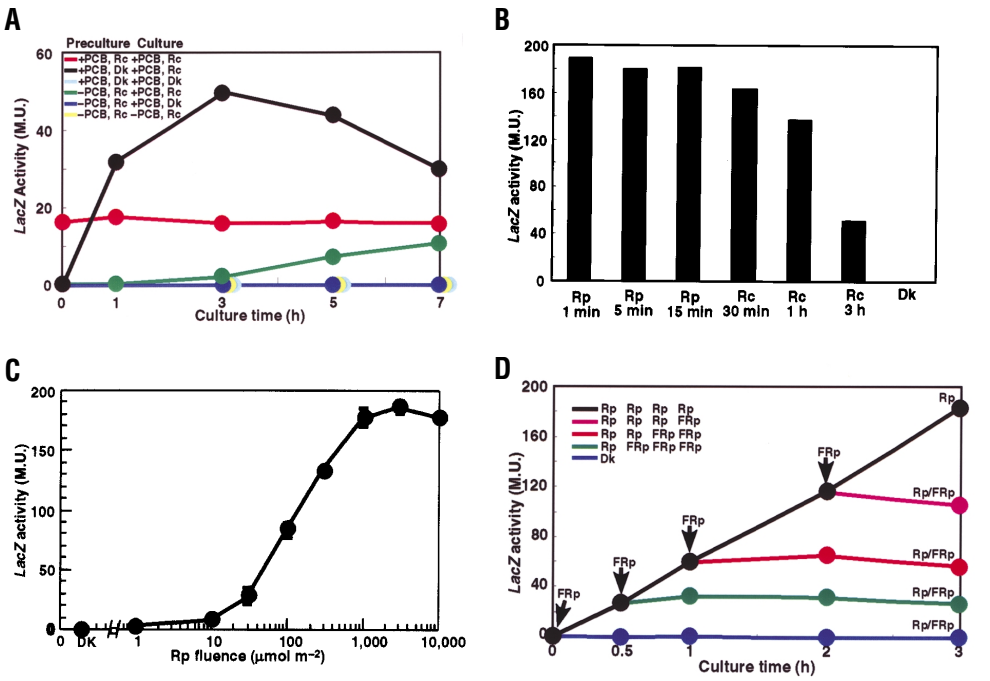

Figure 2. Photoreversible activation of reporter gene expression. (A) Time course of reporter gene expression in yeast cells after various preincubation treatments. Yeast cells (Y187) containing phyB(NT)–GBD and PIF3–GAD were preincubated (preculture conditions inset) with (+) or without (–) 25 μM PCB, either in continuous red light (Rc) or darkness (Dk) for 16 h. These cells were then inoculated into YPD media and cultured further (culture conditions inset). After 0, 1, 3, 5, or 7 h, the cells were collected and LacZ activity was determined. Maximum expression is induced by preincubation of the cells in the presence of PCB in the dark and subsequent transfer to continuous red light (Rc) + PCB. (B) Effect of duration of red light irradiation. Yeast cells co-transformed with phyB(NT)–GBD and PIF3–GAD were preincubated with PCB in darkness. After inoculation into YPD media, the cultures received red light pulses (Rp) and were returned to darkness, or were retained in continuous red light (Rc) or darkness (Dk) (durations, horizontal axis). After 3 h, LacZ activity was measured. Irradiation of 1 min is sufficient to reach the maximum induction of reporter gene expression. (C) Fluence (dose)–response curve of reporter gene expression in yeast. This experiment was done as described in (B) except that a single, short pulse of red light of varying intensity or duration was given at the start of the 3-h culture. (D) Effect of a far-red-light pulse (FRp) in reversing induction of gene expression by red-light pulse (Rp). Yeast cells co-transformed with phyB(NT)–GBD and PIF3–GAD were preincubated in the presence of PCB in darkness. After inoculation into YPD media, the cultures were given either a 5-min Rp (12,000 μmol m^{-2}), or a 5-min Rp followed by a 5-min FRp (18,600 μmol m^{-2}) at 0, 0.5, 1, or 2 h after inoculation. (E) Short-term kinetics of induction, and reversal of induction of gene expression by Rp and FRp. Yeast cells were co-transformed and preincubated as described in (D). After inoculation into YPD media, the cultures were given a 1-min Rp (2,400 μmol m^{-2}) and either were incubated further in darkness for the periods indicated (Rp), or were incubated in darkness for 30 min before being given a 5-min FRp (18,600 μmol m^{-2}) and incubated further in darkness (Rp/FRp) (incubation times, horizontal axis). Yeast cells show Rp-dependent induction of reporter gene expression that can be turned off rapidly by a subsequent FRp. M.U., Miller units.

cells to produce their own chromophore[12], or, in the case of plants, to exploit the naturally produced, endogenous chromophore[16].

We conclude that the system described here has the potential to provide precise, quantitative, spatiotemporal control of conditional expression of selected genes in any light-accessible eukaryotic cell using a universally available, non-invasive, nontoxic inducer.

Experimental protocol

Yeast two-hybrid vector construction. The pGAD424 vector containing the GAL4 activation domain (GAD) was obtained from Clontech (Palo Alto, CA), and the D153 vector containing the GAL4 DNA-binding domain (GBD) was provided by R. Brazas. Full-length phyA and phyB and the N-terminal domain of phyB (amino acids 1–621) were amplified by PCR from full-length cDNAs and cloned into D153, creating phyA(FL), phyB(FL), and phyB(NT), respectively. PIF3–GAD was constructed as described[7].

Yeast two-hybrid plate assay. The yeast strain AH109 (*MATα*, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, 112, *gal4*Δ, *met*$^-$, *gal80*Δ, *URA3*:*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*)[17] was obtained from Clontech. Yeast were transformed according to the Clontech Yeast Protocols Handbook. For the phyA(FL) assay, after heat treatment, the yeast were spread on non-selective synthetic dropout (SD) medium SD(–LW) (Clontech Yeast Protocols Handbook) and incubated for 2 days. The colonies were scraped from plates, resuspended in buffered glycerol solution (32.5% (vol/vol) glycerol, 50 mM $MgSO_4$, and 12.5 mM Tris-HCl, pH 8.0), spread on SD(–LWHA) (Clontech Yeast Protocols Handbook) containing, in addition, 1 mM 3-aminotriazole (3-AT), with or without 25 μM PCB, and incubated under continuous red light (1 μmol m^{-2} s^{-1}) or in darkness for three days. For the phyB(NT) assay, after heat treatment, the cells were cultured for 3 h in darkness in SD(–LWH) medium (Clontech Yeast Protocols Handbook) with or without 25 μM PCB. The transformants were spread on SD(–LWHA) with or without 25 μM PCB, and incubated under continuous red light (1 μmol m^{-2} s^{-1}) or in darkness for three days. PCB was purified as described[18].

Yeast two-hybrid quantitative liquid assay. The yeast strain Y187 (*MATα*, *ura3-52*, *his3-200*, *trp1-901*, *leu2-3*, 112, *gal4*Δ, *met*$^-$, *gal80*Δ, *LYS2*:*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3*, *GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2*, *ura3*:*MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*)[19] was obtained from Clontech. Transformants were grown in SD(–LW) medium with or without 25 μM PCB under continuous red light (40 μmol m^{-2} s^{-1}) or in darkness for 16 h. The culture was inoculated into YPD medium (Clontech Yeast Protocols Handbook) with or without 25 μM PCB under continuous red light (40 μmol m^{-2} s^{-1}) or in darkness. The assay of LacZ activity with *o*-nitrophenyl β-D-galactopyranoside (ONPG; Sigma) as a substrate was done according to the Clontech Yeast Protocols Handbook.

Light sources. Red light (λ_{max}, 660 nm) for longer-term irradiations (Figs 1B, C and 2A, B) was provided by filtered fluorescent tubes[20]. Red light (λ_{max}, 664 nm) and far-red light (λ_{max}, 748 nm) used for pulse irradiations (Fig. 2C–E) was provided by light-emitting diode arrays[8]. All light-sensitive manipulations were done in the darkroom under green safelight (λ_{max}, 550 nm)[20].

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Competing interests statement
The authors declare that they have no competing financial interests.

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An efficient system for the evolution of aminoacyl-tRNA synthetase specificity

Stephen W. Santoro[1], Lei Wang[1], Brad Herberich[1], David S. King[2], and Peter G. Schultz[1]*

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A variety of strategies to incorporate unnatural amino acids into proteins have been pursued[1–5], but all have limitations with respect to technical accessibility, scalability, applicability to *in vivo* studies, or site specificity of amino acid incorporation. The ability to selectively introduce unnatural functional groups into specific sites within proteins, *in vivo*[6,7], provides a potentially powerful approach to the study of protein function and to large-scale production of novel proteins. Here we describe a combined genetic selection and screen that allows the rapid evolution of aminoacyl-tRNA synthetase substrate specificity. Our strategy involves the use of an "orthogonal" aminoacyl-tRNA synthetase and tRNA pair that cannot interact with any of the endogenous synthetase–tRNA pairs in *Escherichia coli*[8–11]. A chloramphenicol-resistance (Cm^r) reporter is used to select highly active synthetase variants, and an amplifiable fluorescence reporter is used together with fluorescence-activated cell sorting (FACS) to screen for variants with the desired change in amino acid specificity. Both reporters are contained within a single genetic construct, eliminating the need for plasmid shuttling and allowing the evolution to be completed in a matter of days. Following evolution, the amplifiable fluorescence reporter allows visual and fluorimetric evaluation of synthetase activity and selectivity. Using this system to explore the evolvability of an amino acid binding pocket of a tyrosyl-tRNA synthetase, we identified three new variants that allow the selective incorporation of amino-, isopropyl-, and allyl-containing tyrosine analogs into a desired protein. The new enzymes can be used to produce milligram-per-liter quantities of unnatural amino acid–containing protein in *E. coli*.

To permit the high-throughput screening of large libraries of synthetase variants using FACS, we constructed an amplifiable fluorescence reporter system. The reporter was designed to allow modulation of screening stringency and was also anticipated to be useful for both visual and quantitative examinations of aminoacylation activity *in vivo*. The fluorescence signal is produced by GFPuv (ref. 12), a variant of green fluorescence protein (GFP) with spectral characteristics suitable for use in FACS, fluorimetry, and visual analysis of living cells. Synthetase-dependent suppression of amber stop codons (TAGs) results in the production of a fluorescence signal. To maximize the reporter's sensitivity, we made it amplifiable (in analogy to other amplifiable intracellular reporters[13,14]) by placing amber codons within the gene for T7 RNA polymerase (RNAP), the product of which drives production of GFPuv. Variable expression of the T7 RNAP gene is achieved using the adjustable *ara*BAD promoter (P_{BAD})[15].

A low-copy plasmid, pREP, was designed to express the genes for T7 RNAP (amber) and GFPuv (Fig. 1A). To develop an optimal fluorescence-based reporter for activity by an orthogonal synthetase in *E. coli*, we inserted a series of 12 T7 RNAP amber mutant variant genes (Fig. 1B) into pREP to create plasmids pREP(1–12). All variant genes encoded a seven–amino acid N-terminal leader sequence (MTMITVH) and one to three TAGs at positions within T7 RNAP predicted to be permissive[16]. To evaluate pREP(1–12) for their ability to report orthogonal synthetase activity, we tested the plasmids with *Saccharomyces cerevisiae* glutaminyl-tRNA synthetase (*Sc*QRS) and glutamine amber suppressor tRNA ($ScQtRNA_{CUA}$), an orthogonal pair for *E. coli*[6]. An ideal reporter should produce strong fluorescence in cells containing wild-type *Sc*QRS and no fluorescence in cells containing an inactive mutant of *Sc*QRS. Each reporter plasmid was introduced into *E. coli* along with either pQ or pQD, compatible plasmids containing the genes for $ScQtRNA_{CUA}$ and either *Sc*QRS or an inactive D291A mutant of *Sc*QRS, respectively. Reporter constructs were evaluated by fluorimetry and flow cytometry of living cells for synthetase-dependent fluorescence. Plasmids pREP(1–12) produced varying levels of synthetase-dependent fluorescence, with the best construct, pREP(10), exhibiting 220-fold greater fluorescence by fluorimetry and ~400-fold greater median fluorescence by cytom-

[1]*Departments of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, SR202, 10550 North Torrey Pines Road, La Jolla, CA 92037.* [2]*Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, CA 94720-1460.*
**Corresponding author (schultz@scripps.edu).*