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Novel Hybrid Input Part Using Riboswitch and Transcriptional Repressor for Signal Inverting Amplifier

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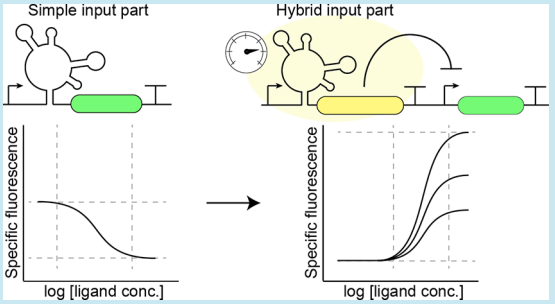
Supporting Information

ABSTRACT: Genetic circuits are composed of input, logic, and output parts. Construction of complex circuits for practical applications requires numerous tunable genetic parts. However, the limited diversity and complicated tuning methods used for the input parts hinders the scalability of genetic circuits. Therefore, a new type of input part is required that responds to diverse signals and enables easy tuning. Here, we developed RNA−protein hybrid input parts that combine a riboswitch and orthogonal transcriptional repressors. The hybrid inputs successfully regulated the transcription of an output in response to the input signal detected by the riboswitch and resulted in signal inversion because of the expression of transcriptional repressors. Dose−response parameters including fold-change and half-maximal effective concentration were easily modulated and amplified simply by changing the promoter strength. Furthermore, the hybrid input detected both exogenous and endogenous signals, indicating potential applications in metabolite sensing. This hybrid input part could be highly extensible considering the rich variety of components.



KEYWORDS: *genetic circuit, input part, riboswitch, transcriptional repressor, coenzyme B_{12}, biosensor*

Genetic circuits are utilized to program cells to perform various tasks.[1,2] A genetic circuit is composed of an input, logic, and output part. The input part controls the expression of the logic part in response to specific signals. Next, the logic part computes the received signal to decide whether to express the output part. For successful construction and application of a genetic circuit, its response to the input signal should be tuned.[3] First, signal amplification is required to sensitively detect the input signal and efficiently trigger the genetic circuit.[4] Furthermore, tuning of the half-maximal effective concentration (EC_{50}) is necessary to meet the requirements of different applications.[5] Finally, the regulation of the genetic circuit needs to be easily converted from the off-type to the on-type and vice versa when designing complex logic.[6]

The most direct method for tuning a genetic circuit is to engineer the input part because it interacts with the input signal and initiates operation of the genetic circuit. In general, simple input parts composed of a single type of biomolecules such as protein or RNA have been employed for genetic circuit construction.[3,7] For example, allosteric transcriptional regulators or riboswitches are typically used as input parts. Engineering simple inputs, however, is not straightforward because the two domains of the input part, the sensor domain for detecting the input signal and transducer domain for propagating the signal, are indivisible and coexist in a single entity. Therefore, it is difficult to modify a specific characteristic of the simple input part while keeping the other undamaged.[8] Although previous studies have altered the properties of simple input parts using rational[4,9] or combinatorial approaches,[5,6,10,11] detailed information regarding the structure and mechanism of input parts remains unclear.

To overcome the conjugated nature of the simple input parts and accelerate the genetic circuit tuning, the two domains can be dissociated to distribute their roles into distinct subparts. For example, two modular genetic parts, one for the input signal detection and another for signal transmission, can be coupled to form a hybrid input part. The consequences of alterations to the hybrid input part should be highly predictable because the original properties of the modular constituents are preserved. Thus, for the hybrid input part, the influence of modification or replacement of a subpart on the feature of the other subpart is negligible. Previously, ligand-inducible aptazymes and transcriptional activators were combined to build RNA−protein hybrid controllers in a eukaryotic cell.[12] Although modularity of the hybrid controller was demonstrated, additional regulatory parts were required to invert the output response to the input signal. Alternatively, a

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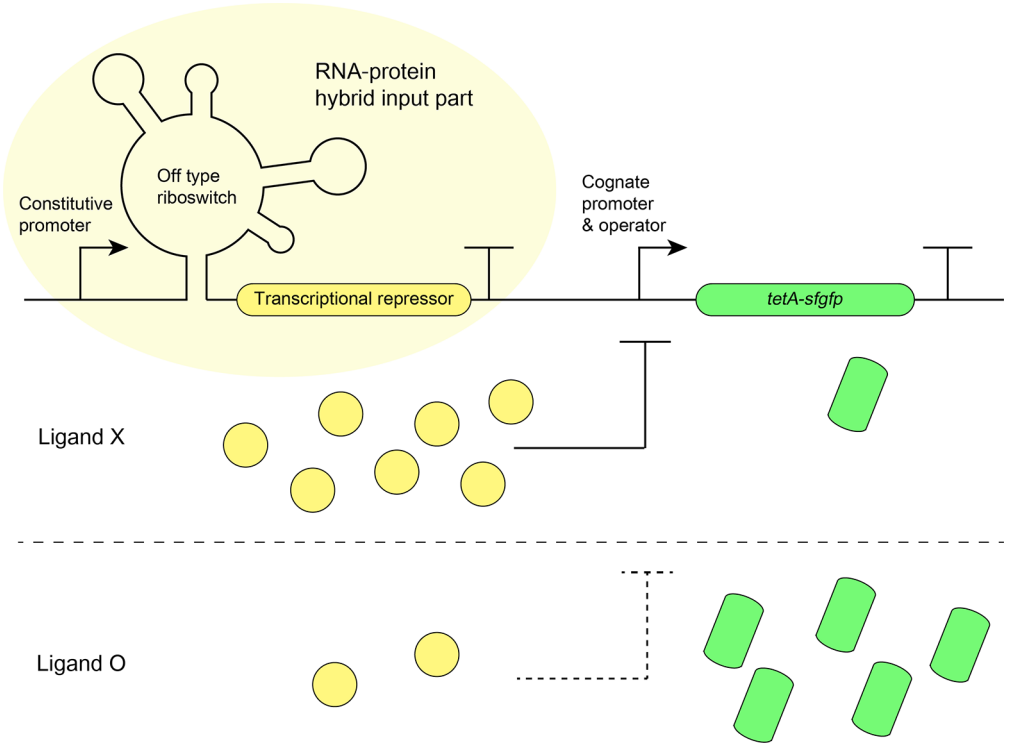

Figure 1. Overall scheme of RNA−protein hybrid input part. The hybrid input part contains riboswitch as a ligand-responsive RNA-based sensor and transcriptional repressor as a protein-based regulator. In the absence of the ligand, the transcriptional repressor is maximally expressed and represses the expression of a reporter gene in downstream of its cognate promoter−operator. On the contrary, in the presence of the ligand, the paucity of the repressor liberates the cognate promoter−operator and the reporter is expressed.

riboswitch and a transcriptional repressor can be combined as a novel input part for prokaryotes (Figure 1). In this architecture, the riboswitch controls the expression of the transcriptional repressor, which in turn, regulates transcription of the output from its cognate promoter. Here, the repressor directly inverts the processed signal from the riboswitch, eliminating the necessity of additional parts. Moreover, the repressor−cognate promoter pair can function as a signal amplifier when its fold-change is larger than the riboswitch part.

In this study, we created RNA−protein hybrid input parts by assembling a natural off-type coenzyme B_{12} riboswitch and two transcriptional repressors. First, we confirmed that these hybrid input parts functioned as signal inverters that convert the input logic from the off-type to the on-type. The expression fold-change and EC_{50} were easily adjusted by modulating the promoter strength for the hybrid inputs. Moreover, the fold-change was controlled by modular replacement of the transcriptional repressor. Finally, the hybrid input part was used to construct a biosensor circuit for detection of intracellular coenzyme B_{12}.

RESULTS AND DISCUSSION

Construction of RNA−Protein Hybrid Input Parts. RNA−protein hybrid input parts were built using a riboswitch and two transcriptional repressors (Figure 1). In this study, we used a coenzyme B_{12} riboswitch from the 5′-untranslated region (5′-UTR) of *cbiA* from *Salmonella typhimurium*[13] as an example. The coenzyme B_{12} riboswitch represses the translation of a downstream gene upon binding of coenzyme B_{12}. Coenzyme B_{12} riboswitches are found in many prokaryotes such as *Escherichia coli*, *S. typhimurium*, and *Bacillus subtilis*, and a consensus sequence between these riboswitches has been identified.[14] Therefore, we utilized the fragment of the *cbiA* 5′-UTR that spans from the beginning of the consensus sequence to the end of the 5′-UTR as an RNA-based sensor component (see Methods). Along with the riboswitch, transcriptional repressors were used to control transcription of an output. Previously, a large number of TetR homologues were cloned and screened for orthogonal transcriptional repression.[15] Among the repressors, LitR and PhlF were chosen because they exhibited high fold-changes upon induction and did not present growth inhibition.

First, we constructed a negative control (NC) that contained only the coenzyme B_{12} riboswitch as an input part. In this architecture, the riboswitch directly regulates translation of a reporter gene (*tetAsfgfp*). This riboswitch effectively repressed gene expression by 7.51-fold when 100 nM of coenzyme B_{12} was supplemented (Figure 2a, b, Table S3). Next, we constructed two genetic circuits containing RNA−protein hybrid input parts composed of the riboswitch and transcriptional repressors. Here, the expression of the final output was transcriptionally regulated by the repressors, which in turn, was controlled by the riboswitch (Figure 1). Transcription of the RNA−protein hybrid input was driven by the BBa_J23108 promoter (strength: 1303 au),[16] the same as used for NC. However, L108 did not grow in liquid media and P108 was insensitive to changes in the coenzyme B_{12} concentration (Figure 2a, b). We hypothesized that the expression level of the repressor was too low, resulting in the excess production of output, severely inhibiting cell growth.[17] Therefore, a stronger promoter, BBa_J23101 (1791 au), was used to rescue the hybrid input circuits from the initial failure. As expected, the hybrid input circuits activated gene expression in response to

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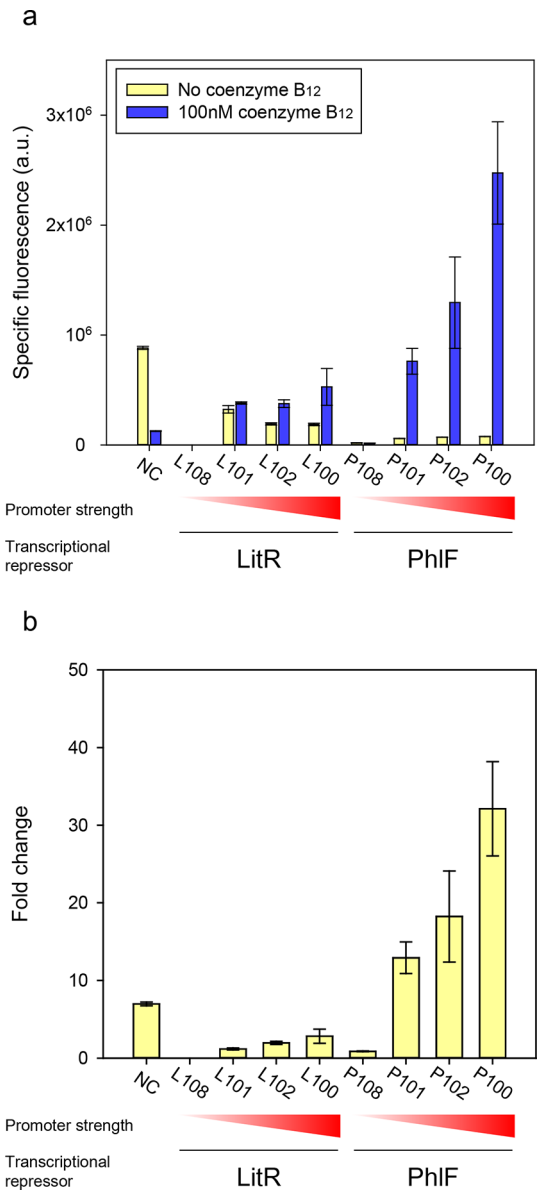

Figure 2. Construction of hybrid input parts. The input part of NC is a coenzyme B_{12} riboswitch, and those of the others are the hybrid parts composed of the riboswitch and transcriptional repressors, LitR or PhlF. (a) Specific fluorescence in the absence (Basal) and presence (Fully induced, 100 nM coenzyme B_{12} added) of the ligand. (b) Fold changes of the gene expression form the hybrid input circuits. Error bars indicate standard deviations from biological triplicates.

coenzyme B_{12} (Figure 2a, b, Table S3). Logic operation of this hybrid input was inverted from that of the NC because the repressor−promoter−operator pairs functioned as NOT gates.[15] Therefore, the hybrid inputs were signal inverters that did not require structural or mechanistic information from the original input part, the coenzyme B_{12} riboswitch, for their construction.

Tuning of RNA−Protein Hybrid Input Parts. The tunability of the dose−response is important because dose−response parameters such as fold-change and EC_{50} should be adjusted to be useful for a variety of applications.[3,18] Because the hybrid input function was easily recovered by changing the promoter strength, we predicted that this promoter modulation can be used as a simple method for tuning the dose−response of a genetic circuit. Accordingly, we assembled additional genetic circuits using stronger promoters to further increase transcription of the hybrid inputs and, in turn, modulate the dose−response parameters of the circuits. Here, we utilized BBa_J23102 (2179 au) and BBa_J23100 (2547 au), the strongest promoters in this artificial promoter series. The differences between the basal and fully induced expression levels drastically increased when strong promoters were utilized (Figure 2a). This promoter-dependent improvement of the input part performance was observed more clearly in terms of the fold-change (Figure 2b). Particularly, P100 exhibited an increase of 32.1-fold, which is the largest value reported to date for coenzyme B_{12}-responsive input parts. These results agree well with previous reports in which the dynamic range and fold-activation were enhanced by creating multiple copies of sensing-actuating events.[17,18] Notably, however, the maximum fold-change that could be achieved principally relies on the properties of the transcriptional repressor because of the modular nature of the hybrid inputs. The fold-changes of LitR and PhlF in their original testbeds were 35 and 193, respectively.[15] This difference was directly reflected in the performance of the hybrid input parts (Figure 2a, b). While the fold changes of the riboswitch and transcriptional repressors are main determinants of the fold change of hybrid inputs, it was suggested that level matching between the two regulatory elements also can affect the fold change.[12] Since the output of the riboswitch is utilized as the input for the cognate promoter of the transcriptional repressor, expression level of the riboswitch should be well-adjusted to fully exploit the fold change of the repressor-regulated promoter. The level matching problem could underlie the fact that the fold changes of LitR and PhlF (35 fold and 193 fold, respectively)[15] are not reflected in exactly the right proportions in the fold changes of the hybrid inputs, L100 and P100 (3.01 fold and 32.1 fold, respectively). It could be reasoned that the expression level range of the riboswitch-regulated transcriptional repressor matched better for controlling PhlF-regulated promoter than for LitR-regulated promoter. Thus, the transcriptional repressor should be chosen carefully when designing new hybrid input parts by taking both fold change and operational range into consideration.

Additionally, we measured the EC_{50} values of the genetic circuits. The EC_{50} represents the operational range of a genetic circuit in which a circuit can discriminate the change in ligand concentration and produce a differential amount of an output. This parameter is extremely important when using a genetic circuit as a genetically encoded biosensor.[3] The hybrid inputs showed increased EC_{50} values compared to the NC (Figure 3a). The EC_{50} further increased slightly when the stronger promoter was utilized for transcription of the riboswitch, as exemplified by the comparison of P101 and P100 (Figure 3b). However, the effect of promoter strength on the EC_{50} was not as large as its effect on the fold changes shown in Figure 2b. It is because the EC_{50} and operational range of a genetic circuit are largely determined by the binding affinities of the genetic circuit components[18] (*i.e.*, binding affinities between coenzyme B_{12} and the riboswitch and between the transcriptional repressors and their cognate promoters of the hybrid inputs). Therefore, the choice of a transcriptional repressor should be a primary focus when tuning EC_{50} and operational range, and promoter strength can be modulated for an additional adjustment. Considering the difficulty in tuning the EC_{50} of

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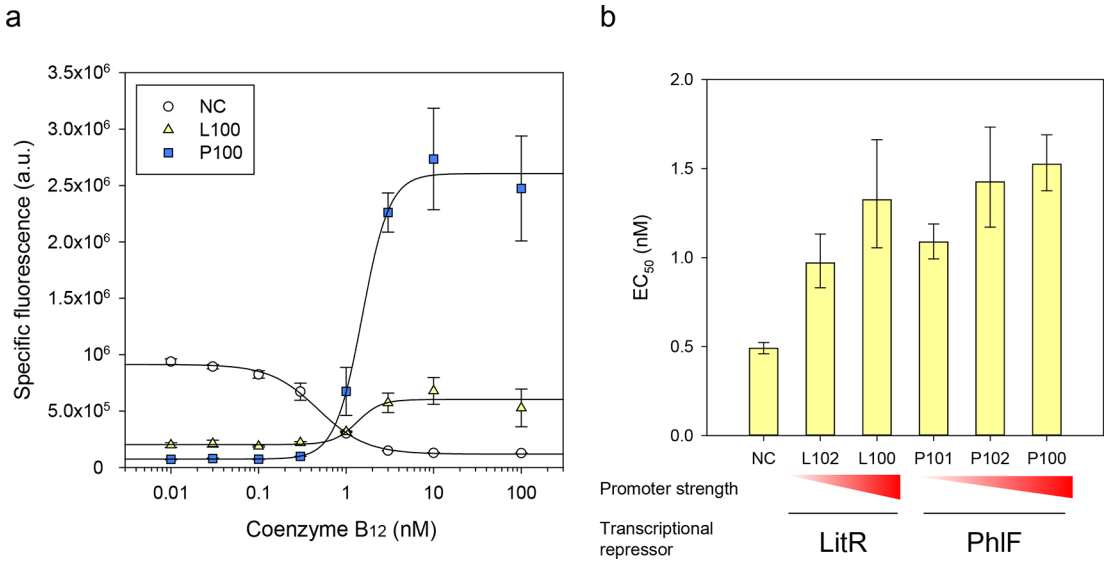

Figure 3. Dose−response parameters of inverting amplifiers with the hybrid input circuits. (a) Dose−response curves of NC, L100, and P100. Error bars indicate standard deviation from biological triplicates. (b) EC_{50} values of the hybrid input circuits. Error bars indicate standard errors from biological triplicates.

RNA inputs,[9,19] using the hybrid input and adjusting the promoter strength may provide a facile tuning method.

Response of the Hybrid Input to an Endogenous Signal. Genetic circuits should respond not only to exogenously transmitted signals but also to endogenously generated signals to enable a wider range of applications. Investigating the intracellular status is crucial for cellular fate programming[20] and metabolic engineering.[3] Accordingly, we investigated whether the hybrid input part can detect a signal generated inside a cell rather than an exogenously added signal. To produce a signal for the hybrid input, coenzyme B_{12}, we exploited the coenzyme B_{12} salvage pathway in *E. coli*. In *E. coli*, *cobC* and *cobUST* code for enzymes that can synthesize coenzyme B_{12} using 1,3-dimethyl-2-phenyl-2,3-dihydro-1*H*-benzoimidazole (DMBI) and ado-cobinamide (Ado-Cbi) as precursors (Figure 4a).[21] The amount of coenzyme B_{12} may be correlated with the amount of Ado-Cbi when excess DMBI is added to the culture media. The P100 strain was cultured with 0.1 mM of DMBI and varying concentrations of Ado-Cbi (0 nM, 1 nM, 10 nM, and 1 μM). The circuit response was measured by fluorescence, and an Ado-Cbi-dependent increase in the response was observed (Figure 4b). The response was saturated when Ado-Cbi was supplemented at concentrations greater than 10 nM. This range agrees with the operational range of the coenzyme B_{12} riboswitch utilized in this hybrid input which also saturates at 10 nM coenzyme B_{12} (Figure 3a). Therefore, the saturation of the response of the hybrid input seems to originate from an innate characteristic of the riboswitch. The saturated fluorescence intensities were similar to those when 1 μM coenzyme B_{12} was added. Moreover, the fluorescence level with 1 nM Ado-Cbi was approximately half of the saturated level, which agrees with the dose−response curve in which the EC_{50} was 1.52 nM (Table S3). These results indicate that the hybrid input circuit successfully detected the intracellular signal. Therefore, the hybrid input circuit can be utilized to monitor the intracellular coenzyme B_{12} concentration for metabolic engineering applications.

Conclusively, we developed RNA−protein hybrid input parts for genetic circuit construction in this study. The hybrid inputs regulated gene expression to the new input signal, coenzyme B_{12}. Furthermore, we demonstrated a straightforward approach for tuning the dose−response parameters of the genetic circuits used for inverting the amplifier. Finally, the hybrid input effectively responded to changes in the intracellular signal. This type of input part can be easily applied for building complex genetic circuits because it controls transcription of the final output in a manner similar to that of conventional genetic circuits. The design of new RNA−protein hybrid inputs would be exceedingly versatile, considering the abundance of RNA sensors and orthogonal transcriptional regulators.

METHODS

Bacterial Strains, Plasmids, and Oligonucleotides. Bacterial strains and plasmids used in this study are listed in Table S1. Sequencing of the plasmids was performed by Cosmogenetech (Seoul, Korea). Oligonucleotides used in this study are listed in Table S2. All oligonucleotides were synthesized by Cosmogenetech.

Genetic Circuit Construction. The hybrid input circuits (pB12ribo_promoter-repressor) were composed of a constitutive promoter, coenzyme B_{12} riboswitch, transcriptional repressor,[15] cognate promoter−operator for the repressor, synthetic 5′-UTR, and reporter (*tetAsfgfp*) (Figure 1).[5,22] The negative control circuit (pB12ribo_J23108-NC) consisted of a constitutive promoter (BBa_J23108),[16] the coenzyme B_{12} riboswitch, and the reporter. The coenzyme B_{12} riboswitch used in this study was a 267-base pair region of the 5′-UTR of *cbiA* in which the consensus sequence of various B_{12} riboswitches was included.[14] To construct the plasmids, each part was PCR-amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs (NEB), Ipswich, MA, USA) and assembled using the Golden Gate assembly method. The riboswitch was obtained by PCR of the genomic DNA of *S. typhimurium* using cbiA_RS_F series primers and cbiA_RS_R series primers. The numbers of the cbiA_RS_F primers denote the constitutive promoters. cbiA_RS_R_RP was used to construct the hybrid input circuits, while

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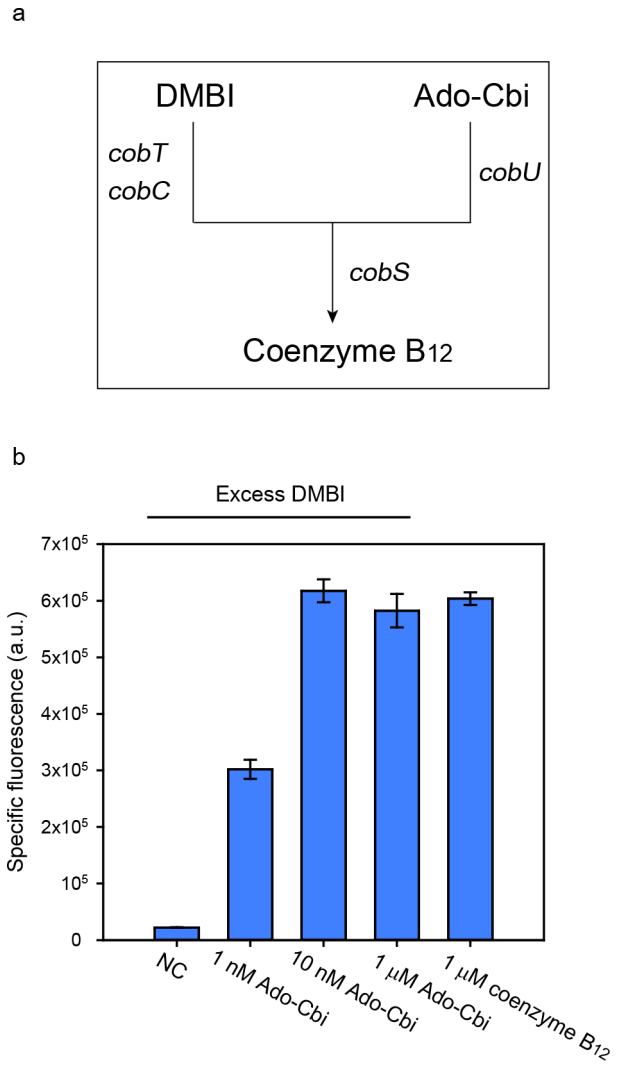

Figure 4. Circuit response to an endogenous signal. (a) Coenzyme B_{12} biosynthesis in *E. coli*. *E. coli* contains cobC and cobUST genes for producing coenzyme B_{12} from DMBI and Ado-Cbi. Therefore, when DMBI and Ado-Cbi were supplemented to the *E. coli* cells, coenzyme B_{12} is produced. (b) Specific fluorescence from the *E. coli* with the hybrid input circuit when supplemented by precursors of different concentration. With excess DMBI, the fluorescence was controlled by the concentration of Ado-Cbi. Error bars indicate the maximum and the minimum values from biological duplicates.

cbiA_RS_R_NC was used as a negative control plasmid. The repressor−promoter−operator cassettes of LitR and PhlF were amplified from pFR-LitR and pRF-PhlF[15] using LitR_F, R, and PhlF_F, R, respectively. Next, the backbone plasmid was constructed by PCR amplification of pTrpRibo[23] with V_rib_rep_F, R for the hybrid input circuits and V_rib_NC_F, R for the negative control. All PCR products were digested with BsaI (NEB) and ligated using Quick Ligase (NEB). The genetic circuits and negative control plasmids were transformed into *E. coli* W3110.

Fluorescence Measurement with Coenzyme B_{12} at Various Concentrations. *Escherichia coli* W3110 strains transformed with the circuit plasmids were cultured overnight in M9 medium with 4 g/L glucose and 34 μg/mL chloramphenicol (CM9). They were diluted with fresh CM9 at a final OD_{600} of 0.05. After cultivation for 8 h at 37 °C with shaking (220 rpm), the culture broth was inoculated with CM9 at a final OD_{600} of 0.05, and coenzyme B_{12} (Sigma-Aldrich, St. Louis, MO, USA) was supplemented at various concentrations (0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 100 nM). The culture broths were incubated for 6 h. Green fluorescent protein fluorescence intensity and OD_{600} were measured using a VICTOR[3] 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). Fluorescence was detected using a 486 nm excitation filter and 535 nm emission filter with a 1 s measurement time. The OD_{600} was measured using a 600 nm filter with a 1 s measurement time. After the measurement, specific fluorescence was calculated by normalizing the fluorescence intensity to the OD_{600}. All cultures were performed in biological triplicate.

Fitting of Dose−Response Curves and Calculation of EC_{50}. Dose−response curves of the hybrid input circuits were plotted using the specific fluorescence. The plots were fitted using a four-parameter logistic equation: Specific fluorescence = Min. + (Max. − Min.)/(1 + $10^{(\log(EC_{50})-\log(\text{coenzyme } B_{12}))\times(\text{Hill coeff.})}$). EC_{50} values were calculated from the fitting results. Fitting of the dose−response curves and the calculation of the EC_{50} values were conducted using SigmaPlot software (Systat Software, Inc., San Jose, CA, USA).

Validation of the Circuit Response to Endogenously Produced Coenzyme B_{12}. The response of the hybrid input circuit to an endogenous signal was investigated. Exploiting the coenzyme B_{12} salvage pathway (*cobC* and *cobUST*) in *E. coli*, the intracellular coenzyme B_{12} concentration was adjusted by adding two precursors, 1,3-dimethyl-2-phenyl-2,3-dihydro-1*H*-benzoimidazole (DMBI) (Sigma-Aldrich) and ado-cobinamide (Ado-cbi) (Sigma-Aldrich). For a seed culture, a single colony of the P100 strain was inoculated into 3 mL of CM9. After 12-h cultivation at 37 °C with shaking (220 rpm), the seed culture was inoculated into fresh CM9 at an OD_{600} of 0.05. When the OD_{600} reached 0.8−1.0, the culture broth was diluted again with fresh CM9 at an OD_{600} of 0.05. DMBI (0.1 mM) and Ado-cbi at various concentrations (0 nM, 1 nM, 10 nM, and 1 μM) were initially added to change the intracellular coenzyme B_{12} concentration. After 6 h of cultivation, OD_{600} and GFP fluorescence were measured using a VICTOR[3] 1420 Multilabel Counter (PerkinElmer). Specific fluorescence was calculated by normalizing the fluorescence intensity to the OD_{600}. All cultures were performed in biological duplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00213.

Strains and plasmids used in this study (Table S1); Primers used in this study (Table S2); Dose−response parameters of the hybrid input circuits (Table S3); Dose−response curves of the hybrid input circuits (Figure S1) (PDF)

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Author Contributions

[§]S.J. and S.J. contributed equally to this work. S.J., S.J., and G.Y.J. designed the study. S.J., S.J., M.H.N. performed the experiments. S.J., S.J., M.H.N., H.G.L., and G.Y.J. analyzed the data and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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