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Synthetic Gene Involving Azobenzene-Tethered T7 Promoter for the Photocontrol of Gene Expression by Visible Light

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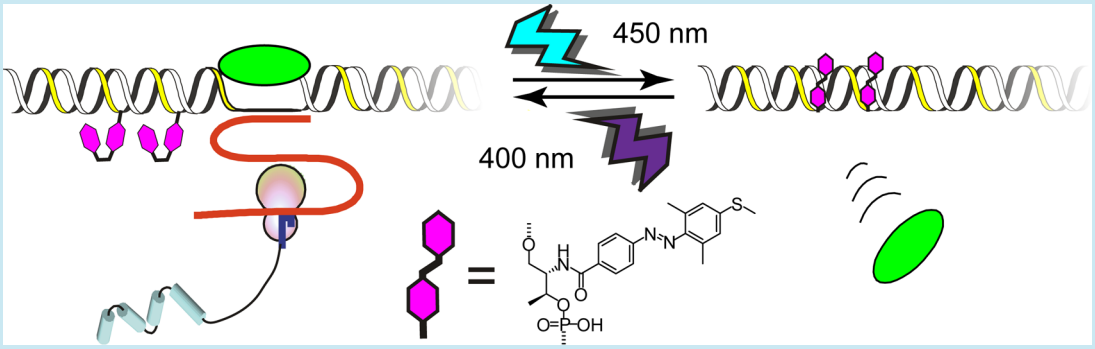
Supporting Information



ABSTRACT: In the present study, we demonstrate photoregulation of gene expression in a cell-free translation system from a T7 promoter containing two azobenzene derivatives at specific positions. As photoswitches, we prepared azobenzene-4′-carboxlyic acid (Azo) and 2,6-dimethylazobenzene-4′-carboxylic acid (DM-Azo), which were isomerized from *trans* to *cis* upon irradiation with UV light (λ < 370 nm), and 4-methylthioazobenzene-4′-carboxylic acid (S-Azo) and 2,6-dimethyl-4-(methylthio)azobenzene-4′-carobxylic acid (S-DM-Azo), which were *cis*-isomerized by irradiation with 400 nm visible light. Expression of green fluorescent protein from a promoter modified with S-Azo or S-DM-Azo could be induced by harmless visible light whereas that from a promoter modified with Azo or DM-Azo was induced only by UV light (340−360 nm). Thus, efficient photoregulation of green fluorescent protein production was achieved in a cell-free translation system with visible light without photodamage.

KEYWORDS: *azobenzene, visible light, transcription, gene expression, photoactivated,* D*-threoninol scaffold*

Artificial regulation of gene expression using compounds such as siRNA is utilized for investigation of the mechanisms of the biological molecules and has potential for therapeutic manipulation of gene expression.[1,2] Spatiotemporal control of gene expression is useful for elucidation of the functional roles of specific targets. Photochromic compounds that undergo reversible conformational changes upon photoirradiation have been used as control element for manipulation of conformation or activity of biomolecules.[3−14] For example, azobenzene can be reversibly photoisomerized between the *trans* and *cis* forms either by ultraviolet (UV: 310−370 nm) or visible light (λ > 400 nm) irradiation. We have previously reported that insertion of azobenzene within the strand of a DNA duplex can induce reversible formation and dissociation of the duplex upon light irradiation.[15−17] In this system, planar *trans*-azobenzene intercalates between the base-pairs and stabilizes the DNA duplex, whereas the nonplanar *cis*-azobenzene destabilizes the duplex due to the steric hindrance. Azobenzene-dependent regulation of duplex formation enables construction of DNA-based photon-driven nanodevices, such as light-powered tweezers or DNAzymes, without waste production.[18,19]

We recently proposed that use of an azobenzene-tethered DNA could enable on−off photoswitching of transcription as schematically illustrated in Figure 1a.[20,21] This regulation is based on not photoregulated hybridization but on a local structural change of DNA duplex around the azobenzene induced by photoisomerization. Far below the melting temperature, a DNA duplex does not dissociate even when the azobenzene is in *cis* form but local structure of duplex around azobenzene is altered. This structural change significantly affects binding behavior of T7 RNA polymerase (RNAP) to the photoresponsive T7 promoter. The *trans*-azobenzene isomerized by visible light irradiation terminates transcription by inhibiting binding of RNAP to T7 promoter, whereas the *cis*-isomerization by UV light irradiation facilitated

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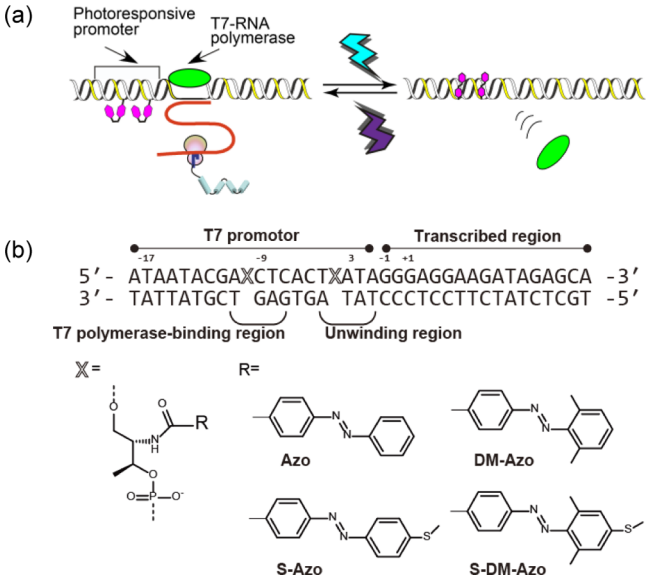

Figure 1. (a) Schematic illustration of the reversible photoswitching of transcription using an azobenzene-tethered T7 promoter. (b) Sequences of photoresponsive T7 promoters and chemical structures of azobenzene derivatives used in this study are shown. Photoswitch molecules (X) were inserted at the indicated positions in the T7 promoter.

the binding of RNAP and melting the unwinding region, switching on the transcription. Therefore, an azobenzene-based light-responsive system can convert a photosignal directly to RNA sequence, and this signal can be further translated to protein. Importantly, with a photoinducible system, there are no waste products to contaminate the reaction system.

For application to the synthetic or molecular biology, however, we cannot use strong UV light for isomerization of azobenzene because UV light damages the biological molecules. Indeed, protein expression in *in vitro* system was greatly reduced upon irradiation of at around 360 nm due to photodamage (SI Figure S1). In order to overcome this disadvantage of conventional azobenzene, we recently developed new azobenzene derivatives that can be isomerized by visible light (λ > 400 nm). One derivative has a methylthio group at 4-position of azobenzene: 4-methylthioazobenzene (S-Azo).[22] Modification of azobenzene at *para*-position with an electron donating group results in a bathochromic shift of absorption maximum to visible light region (~400 nm); *trans*-to-*cis* and *cis*-to-*trans* photoisomerization can be carried out at 400 and 450 nm, respectively. This modification reduced thermal stability of the *cis* form, however.[23,24] We also have found that introducing methyl groups at two *ortho* positions of the distal benzene ring (4′-carboxy-2,6-dimethylazobenzene) suppressed thermal isomerization of *cis*-to-*trans* form.[25] By combining methylthio-modification at *para*-position with methylation of two *ortho*-positions of azobenzene, we synthesized 2,6-dimethyl-4-(methylthio)azobenzene-4′-carboxylic acid (S-DM-Azo), which was reversibly isomerized by only visible-light irradiation (λ > 400 nm); the *cis* form is thermally stable.[26] In the present study, we demonstrate on−off photoswitching of transcription and subsequent translation only by visible-light irradiation in a cell-free system without photodamage.

To construct the photoresponsive transcription system, we synthesized template DNA with azobenzenes-installed T7 promoter. Figure 1b shows the T7 promoter region and the site of azobenzene modification;[20,21] two azobenzenes were inserted into the RNA polymerase recognition region and the unwinding region through D-threoninol linkers, as described previously.[21] Transcription reactions were performed *in vitro*. DNA template with the photoresponsive T7 promoter was mixed with T7 RNA polymerase and [α-^{32}P]ATP after *cis*-isomerization by light-irradiation at 340 nm for Azo and DM-Azo or at 400 nm for S-Azo and S-DM-Azo or in the dark conditions to ensure adoption of the *trans* form. Note that UV light irradiation does not alter transcription efficiency.[20,21] After the reaction, 17-nucleotide RNA products were separated by PAGE analyses (Figure 2a) and conversion efficiencies from

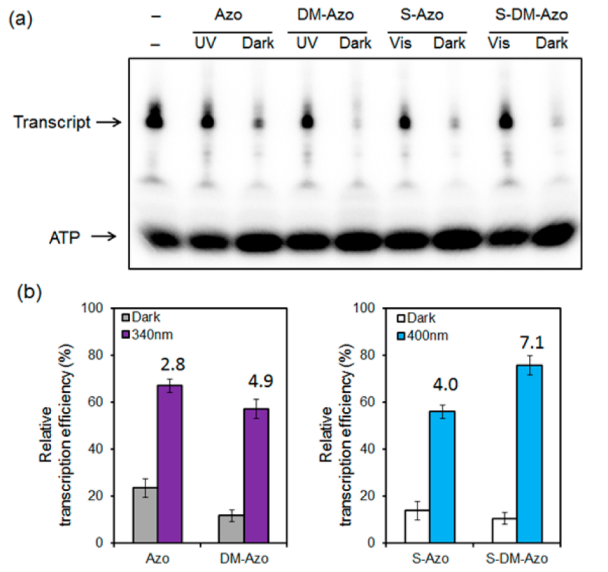

Figure 2. Photocontrol of the transcription reaction with the T7 promoter modified with Azo or Azo derivatives. (a) PAGE profiles of RNA products. Templates tethered Azo or Azo derivatives were heated to induce *trans* isomerization before the reaction. The *cis* form was induced by either UV light (340 nm) or visible light (400 nm). (b) Relative amount of RNA product were shown in bar graph.

ATP to transcripts were determined based on the signal intensity (Figure 2b). Templates modified with Azo or DM-Azo produced more transcripts after irradiation at 340 nm. In both cases, relative transcription efficiencies were around 60% of transcripts from unmodified template, whereas relative transcription efficiencies of transcripts with *trans*-DM-Azo or *trans*-Azo (under dark conditions) were around 20%.

The photoregulation efficiency (α) of transcription was defined as the ratio of the amount of transcript after *cis* isomerization with respect to amount obtained in *trans* form (Figure 2b). The α values for the conventional Azo and for DM-Azo promoters were 2.8 and 4.9, respectively. Thus, photoregulation of transcription by irradiating with UV light was obtained. However, these photoswitches are not activated with visible light (λ > 400 nm) because these azobenzenes do not isomerize to *cis* form with visible light (SI Figure S2). Unlike Azo or DM-Azo, S-Azo or S-DM-Azo tethered T7 promoters are activated by irradiation with 400 nm light. The S-DM-Azo-modified promoter yielded significantly more transcript than did the S-Azo-modified promoter (>70%) after light irradiation whereas transcription was suppressed more efficiently for *trans*-S-DM-Azo (around 10%) than for *trans*-S-

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Azo (around 15%). The α values of S-DM-Azo and S-Azo with irradiation at 400 nm were 7.1 and 4.0, respectively. Thus, S-DM-Azo was particularly suited for efficient photoregulation by visible light stimulation. As described above, *cis*-S-DM-Azo is thermally much more stable than *cis*-S-Azo; the half-life of *cis*-S-DM-Azo in the single-stranded DNA is 6.4 h at 60 °C (36.5 h at 37 °C) under buffer condition whereas that of S-Azo is only 0.4 h.[26] During transcription reaction, *cis*-S-Azo gradually isomerized to the *trans* form which reduced transcription yield.

We next examined the effect of photoswitching of transcription by irradiating first with 400 nm and then with 450 nm light with the S-DM-Azo-modified T7 promoter. The reaction was started in the dark condition so that transcription levels were initially low (Figure 3). After irradiation with 400 nm

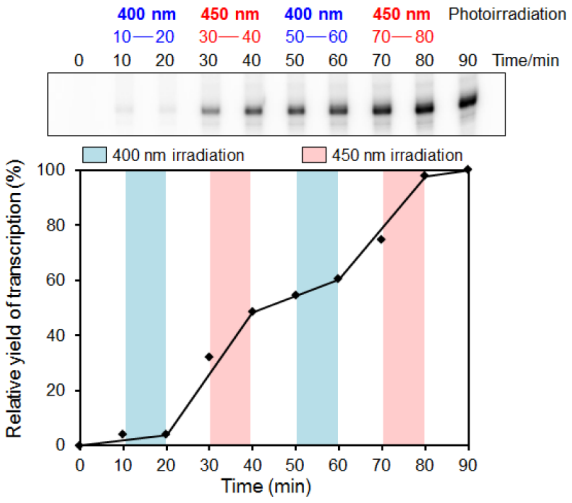

Figure 3. Photoswitching of transcription from an S-DM-Azo-modified T7 promoter by alternate irradiation with 400 and 450 nm light. The reaction mixture was subjected to photoirradiation with 400 nm for 10 min after 10 and 50 min of reaction time. It was subjected to irradiation with 450 nm light for 10 min after 30 and 70 min of reaction time. RNA products were separated by PAGE (top). The relative yield of transcription based on the conversion efficiencies of ^{32}P from ATP to RNA was plotted (bottom). Conversion efficiency at 90 min was set to 1.0.

light, transcription was efficiently induced. Subsequent irradiation at 450 nm reduced RNA production. Thus, the alternate exposure of the reaction mixture with 400 and 450 nm light allowed on−off switching of the transcriptional response.

We further analyzed whether transcription and following translation reaction could be regulated by the photoresponsive T7 promoter modified with S-DM-Azo. We prepared a green fluorescent protein (GFP) reporter gene driven by the photoresponsive T7 promoter essentially as described[27] with some modifications (Figure 4 and SI Figure S3). T7-promoter sequences modified with Azo or S-DM-Azo used as forward primers (Primer L) to produce the overhanging DNA (I) including *GFP*-coding sequence from pQBI-T7-GFP plasmid (Qbiogene) (SI Figure S3b). Elongation from Primer R along the sense strand halts before the azobenzene residue. The PCR products were annealed and ligated with DNA of sequence complementary to the promoter region (Com24). The resulting long double-stranded DNAs (dsDNA; II in Figure 4 and SI Figure S3c) with Azo (Azo-GFP) or S-DM-Azo (SDM-Azo-GFP) were used as templates for *in vitro* transcription/translation.

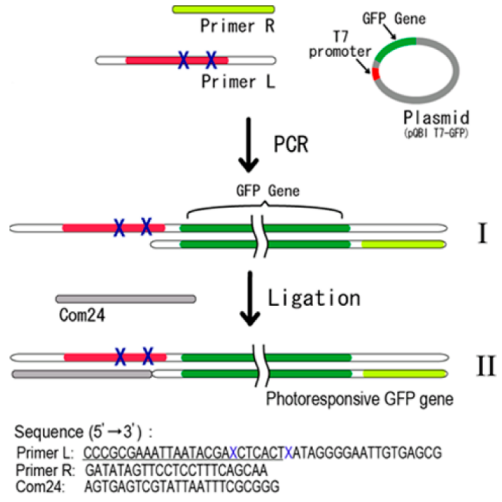

Figure 4. Schematic representation of reporter gene containing GFP-coding DNA under the control of the T7 promoter modified with photoresponsive molecules.

We first analyzed GFP production from *cis*-Azo-GFP and *cis*-SDM-Azo-GFP. Prior to reaction initiation, dsDNAs were irradiated with 340 or 400 nm light for 10 min to isomerize the photoswitches to the *cis* forms. Reaction reagents were added and the mixture was incubated at 37 °C for a predetermined time and then cooled to 4 °C to stop the transcription−translation reaction. Subsequently, the protein products were incubated for 1.5 days at 4 °C to enable refolding of GFP. The relative GFP expression level was calculated based on the fluorescence intensity (ex. 474 nm, em. 508 nm) (Figure 5). Since the *trans*-to-*cis* isomerization was conducted before mixing the transcription−translation reagents, GFP was efficiently produced without photodamage *in vitro* translation system. As we expected, the *cis* forms of S-DM-Azo and Azo facilitated transcription and subsequent translation; amount of GFP increased almost proportionally to incubation time at 37

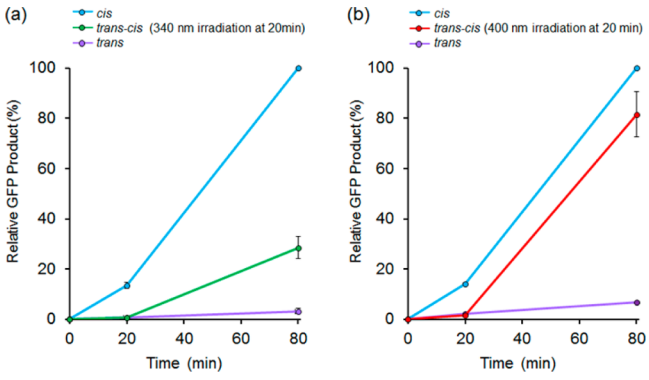

Figure 5. Relative protein production from (a) Azo-GFP and (b) SDM-Azo-GFP were calculated on the basis of GFP fluorescence after excitation at 474 nm. The GFP reporter genes were irradiated at 340 nm for 5 min for Azo or 400 nm for 10 min for S-DM-Azo, respectively, to induce the *cis* conformation. To obtain the *trans* conformation samples were irradiated with 450 nm light for 5 min. Subsequently, the GFP expression vectors were mixed with the reaction reagent to start the GFP translation reaction. After 20 or 80 min, the reactions were stopped by mixed with loading buffer and incubated at 4 °C for protein folding. To switch the conformation from *trans* to *cis* during the reaction, reaction mixtures were irradiated at 340 nm for Azo or 400 nm for S-DM-Azo after 20 min.

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°C after *cis* isomerization as depicted with blue lines in Figure 5. In contrast, when the photoswitches were in *trans* form, induced by irradiating with 450 nm for 5 min at 60 °C prior to addition of reaction mix, no GFP was produced (Figure 5, purple lines). The ratios of GFP produced when the azobenzene derivatives in *cis* form with respect to *trans* form were 14.7 for SDM-Azo-GFP and 31.2 for Azo-GFP. This translation ratio was far larger than that for transcription ratio from GFP reporter gene (SI Figure S4). Note that, in the cell-free system, both *cis*-Azo-GFP and *cis*-SDM-Azo-GFP allow GFP production, and the amount of GFP increased almost linearly with incubation time after *cis* isomerization.

Next, we performed off-to-on photoswitching of GFP expression with photoresponsive GFP genes by irradiating the cell-free system directly. First, both Azo-GFP and SDM-Azo-GFP were switched off by irradiating with 450 nm light. Little GFP was produced at 20 min after incubation from the reporter genes (Figure 5a, green line and Figure 5b, red line). The reaction mixture containing *trans*-Azo-GFP was irradiated with 340 nm UV light for 10 min. After incubation for an additional 60 min, a distinct increase in GFP production was observed (Figure 5a, green line). However, the amount of GFP produced was far smaller than that expected from 60 min incubation. Undoubtedly, this was due to damage of the enzymes in the expression system by UV light (SI Figure S1). In contrast, SDM-Azo-GFP showed remarkable off-to-on switching. When the SDM-Azo-GFP was irradiated with 400 nm light, GFP production after 60 min was almost that of a 60 min reaction in the absence of photoswitching (Figure 5b, red line).

In conclusion, we constructed a photoresponsive *GFP* gene using a promoter modified with S-DM-Azo that was regulated by harmless visible-light irradiation. The S-DM-Azo system was superior to the Azo system for biological application as a visible-light switch does not harm reaction components as UV does. We believe that T7 expression system involving S-DM-Azo will be useful *in vivo* application such as phototriggered expression of target protein or release of shRNA.[30] Further application of photoresponsive gene to liposome-based artificial cells for synthetic biological studies is also expected.

METHODS

Materials. Oligonucleotides consisting of only native bases were supplied by Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.). We synthesized phosphoramidite monomers modified with Azo, DM-Azo, S-Azo, or S-DM-Azo, as described previously.[22,26,28,29] DNAs modified with Azo, DM-Azo, S-Azo, or S-DM-Azo were obtained from Nihon Techno Servise Co. Ltd. (Tsukuba, Japan) or synthesized on an ABI-3400 DNA synthesizer (Applied Biosystems, CA, U.S.A.) with conventional and dye-carrying phosphoramidite monomers. After the recommended workup, the synthesized DNAs were purified by HPLC on a reverse-phase column. Concentrations of all oligonucleotides were determined by UV/visible spectroscopy analysis. T7 RNA polymerase was purchased from TAKARA Bio, Inc. (Japan). Phusion polymerase was purchased from New England Biolabs. PURESYSTEM classic II was purchased from WAKO Pure Chemical Industries, Ltd. (Japan).

Photoisomerization of Azobenzene. A xenon light source (MAX-301, Asahi Spectra Co., Ltd. Tokyo, Japan) equipped with interference filters was used for photoisomerization. The following filters were used: 340 nm (half bandwidth 9 nm) centered at 340.5 nm; 360 nm (half bandwidth 10 nm) centered at 360 nm; 370 nm (half bandwidth 12 nm) centered at 369.0 nm; 390 nm (half bandwidth 10 nm) centered at 390.0 nm; 400 nm (half bandwidth 11 nm) centered at 398.5 nm; 450 nm (half bandwidth 9 nm) centered at 449.5 nm. The sample solution was added to a cuvette, and the temperature of light irradiation was controlled by using a programmable temperature controller (JASCO, Tokyo, Japan).

Transcription Catalyzed by T7 RNA Polymerase. To isomerize to *cis* form, the template for transcription reaction (Figure 2) was irradiated at 340 nm for 5 min for Azo and DM-Azo and irradiated at 400 nm for 10 min for S-Azo and S-DM-Azo. To induce *trans* isomerization of these azobenzene derivatives, the templates were heating at 80 °C for 10 min. Then, the templates were dissolved in reaction buffer containing 0.5 mM each NTP, 2 μCi of [^{32}P]ATP[αP], 40 mM Tris-HCl (pH 8.0), 2 mM spermidine, 5 mM dithiothreitol, 24 mM $MgCl_2$, and 2 mM NaCl. After mixing with 50 U of T7 RNA polymerase, the reaction samples were incubated at 37 °C for 1 h. Then, 5 U of DNase I was added, and samples were incubated for 15 min at 37 °C. For photoswitching of transcription with S-DM-Azo involving T7 promoter, alternate irradiation with 400 and 450 nm light was performed. Before the reaction, the templates were heated at 80 °C to induce *trans* isomerization of S-DM-Azo in the template. The reaction was started with the template in the dark. Subsequently the reaction mixture was irradiated for 10 min with 400 nm light after 10 min, 450 nm after 30 min, 400 nm after 50 min, and 450 nm after 70 min of reaction time. Reactions were quenched by adding 21 μL of loading buffer containing 80% formamide, 50 mM EDTA, and 0.025% bromophenol blue. A 5.5-μL aliquot of the mixture was subjected to electrophoresis on a 10% denaturing polyacrylamide gel containing 8 M urea at 750 CV for 45 min. After exposure to an imaging plate (BAS-MS2340; Fujifilm, Tokyo, Japan) radioisotopic images were analyzed with an FLA-7000 bioimaging analyzer (Fujifilm, Japan).

GFP Expression Template Including Azo or S-DM-Azo. We prepared GFP expression systems driven by a photoresponsive T7 promoter (SI Figure S2a). PCR amplification reactions were performed with pQBI-T7-GFP plasmid (Qbiogene), forward primers (Primer L) possessing the Azo- or S-DM-Azo-containing T7-promoter sequence, and Primer R to produce the overhanging DNA (I) including *GFP*-coding sequence. Note that elongation from Primer R along the sense strand stops before the azobenzene residue. PCR reaction conditions were Primer L (0.4 μM), Primer R (0.4 μM), pQBI-T7-GFP (1 ng/μL), dNTP (0.16 mM), Phusion high-fidelity DNA polymerase (0.02 U/μL) in polymerase reaction buffer. We performed 30 PCR cycles: 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 45 s. The reaction solution was mixed with loading buffer containing EDTA (50 mM) and bromophenol blue (0.025%). The mixture was subjected to electrophoresis on an agarose gel (1%) at 100 V for 60 min (SI Figure S2b). The PCR products were annealed and ligated with 5′ phosphorylated DNA complementary to the T7 promoter region (Com24). Before annealing, Azo or S-DM-Azo was *cis* isomerized by light-irradiation for 10 min at 60 °C with 340 or 400 nm light, respectively. Then, Com24 was mixed with the PCR product (I) and kept at 25 °C. Subsequently, the sample was mixed with reaction reagent containing T4 DNA ligase and incubated at 25 °C for 1.5 days. In order to confirm successful ligation, we used FAM-labeled Com24. Aliquots were added to loading buffer containing 80% formamide, 50 mM EDTA, and 0.025% bromophenol blue and subjected to electrophoresis on

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a 10% denaturing polyacrylamide gel containing 8 M urea at 900 CV for 40 min. As shown in SI Figure S2c, we detected a low mobility band that corresponded to ligation product (II) by using FLA-7000 bioimaging analyzer. Reaction conditions were 0.05 μM PCR product (I), 0.2 μM Com24, and T4 DNA ligase in NEB T4 DNA ligase reaction buffer.

Photoisomerization of Azobenzene and Its Derivatives. The mixture of PURESYSTEM-sol A and PURESYSTEM-sol B was prepared before starting the translation reaction. The GFP-expression template was irradiated at 450 nm for 5 min at 60 °C for isomerization to the *trans* form of Azo or S-DM-Azo. To obtain the *cis* form of Azo or S-DM-Azo, Azo-GFP or SDM-Azo-GFP were irradiated with 340 or 400 nm for 10 min at 60 °C, respectively. After light irradiation, the template DNA was mixed with PURESYSTEM reagent and incubated at 37 °C. The reaction mixture was sampled after 20 and 80 min of reaction time and subsequently incubated at 4 °C to allow proper folding of GFP for 1.5 days. GFP expression was monitored by measurement of fluorescence (ex. 474 nm, em. 508 nm) using a plate reader (Enspire and ARVO, PerkinElmer Inc.). To perform the photoswitching, reactions were photoirradiation for 10 min with 340 nm for Azo or 400 nm for S-DM-Azo after 20 min of reaction time.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−4 are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(30) In order to use photoresponsive gene in living cell, circularized plasmid having photoresponsive T7 promoter should be necessary.