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Light-Activated Gene Expression System Using a Caging-Group-Free Photoactivatable Dye

Tatsuki Nonomura, Masafumi Minoshima, and Kazuya Kikuchi**

Abstract: Optical regulation of transcription using chemical compounds is an effective strategy to manipulate gene expression spatiotemporally. Conventional caging approaches with photoremovable protecting groups may require intense UV-light exposure and release potentially toxic byproducts. To address these problems, here we developed a light-mediated transcriptional regulation system by combining a caging-group-free photoactivatable dye PaX_{560} and a multidrug-binding transcriptional regulator QacR. The cationic dye generated from PaX_{560} through traceless photoconversion bound QacR and reduced its repressor function, resulting in transcriptional activation. Importantly, this system allowed transcriptional activation with a large dynamic range under mild visible light exposure and simultaneous detection of the state of the photoactivated effector. This module was integrated into the T7 RNA polymerase expression system to demonstrate light-activated transcription in vitro and in living cells.

Introduction

Regulation of biological processes using photochemical compounds is a crucial method to investigate cellular activities, as it offers high spatiotemporal resolution without the need for intricate protein engineering. In particular, optical control of transcription has attracted attention because of its versatility in modulating gene expression, one of the most fundamental biological phenomena.[1] Over the past decades, many efforts have been made to develop light-mediated transcriptional regulation systems by installing photoremovable protecting groups (PPGs) on biologically active molecules such as small compounds,[2–10] nucleic acids,[11–21] and proteins.[22,23] PPGs block the original function by binding to the target biomolecule and caging the site required for the biological function of the target, which is restored upon exposure to light that uncages the molecule.[24,25] Several PPGs, including *o*-nitrobenzyl and coumarin derivatives, have been developed and employed for photo-regulation of transcription. Although this approach is commonly used to achieve transcriptional regulation, there exist some limitations. PPGs often require intense and long duration of UV light exposure due to their low uncaging efficiency, which may cause undesirable damage to the cells.[1,25] In addition, the resulting photoproducts can be toxic. For example, commonly used *o*-nitrobenzyl PPGs contain highly reactive nitroso groups.[25] Finally, the states of effector molecules, i.e., whether they are caged or uncaged, cannot be distinguished visually, making it impossible to monitor the activity state of the effector molecules. Hence, quantitation of transcriptionally activated cells is a significant challenge, unless the target genes encode specific proteins that produce optically detectable signals, such as green fluorescent protein, luciferase, and β-galactosidase.[2–7,9,10,13–16,20–23] Those limitations confine the application of conventional PPGs to photo-regulation of genes in general. Therefore, it is highly desirable to develop an innovative optochemically controlled transcription module that can be activated by low-intensity light exposure that allows simultaneous detection of the activity state of the effector molecule.

To address the technological shortcomings, here we report a new class of optical transcriptional regulation system in which a photoactivatable fluorophore functions as an effector for a transcription factor (Figure 1). Among a plethora of photoactivatable dyes, we focused on the silicon-bridged xanthone PaX_{560} recently developed by Butkevich, Hell, and co-workers.[26] This photoactivatable dye is capable of switching from a non-fluorescent Si-xanthone, PaX_{560}, to

[*] T. Nonomura, Dr. M. Minoshima, Prof. Dr. K. Kikuchi
Department of Applied Chemistry, Graduate School of Engineering, Osaka University
2-1, Yamadaoka, Suita, Osaka, 5650871 (Japan)
E-mail: kkikuchi@mls.eng.osaka-u.ac.jp
minoshima@mls.eng.osaka-u.ac.jp
Dr. M. Minoshima
JST, PRESTO
2-1, Yamadaoka, Suita, Osaka, 5650871 (Japan)
Prof. Dr. K. Kikuchi
Immunology Frontier Research Center (IFReC), Osaka University
2-1, Yamadaoka, Suita, Osaka, 5650871 (Japan)
T. Nonomura
Present address: Center for Translational Cancer Research, Institute of Biosciences and Technology, Texas A&M University,
2121W Holcombe Blvd, Houston, TX-77030 (USA)

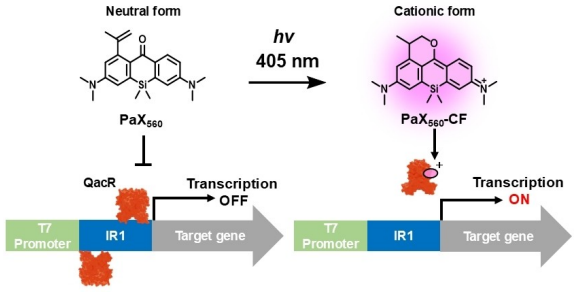

Figure 1. Schematic of the light-activated transcription system using the photoactivatable dye PaX_{560} as an effector molecule and the IR1 operator-binding factor QacR as a transcription regulator.

a highly fluorescent Si-pyronine, PaX_{560}-Closed Form (CF), upon mild light illumination. It has been reported that PaX_{560} is activated by visible light (405 nm) and exhibits high photoactivation efficiency ($\Phi_{Act} \times \varepsilon_{405nm} = 1410$).[26] Furthermore, the photoconversion occurs without releasing any byproducts. Owing to its light-induced fluorogenic nature, PaX_{560} has been applied to live-cell imaging and super-resolution imaging.[26,27] In addition to its fluorogenicity, PaX_{560} undergoes photoconversion into a cationic Si-pyronine from a neutral Si-xanthone. We employed the positive charge acquisition to optically regulate the activity of a multidrug-binding transcriptional regulator, QacR. QacR found in *Staphylococcus aureus* is classified as a TetR-family transcriptional regulator (TFTR).[28] It functions as a transcription repressor that regulates gene expression of an antibiotic efflux pump by interfering with an operator sequence, IR1.[29,30] The repression activity of QacR is mitigated upon binding of a range of cationic lipophilic dyes, such as rhodamine 6G, pyronin Y, ethidium, and crystal violet, resulting in transcriptional activation of the associated genes.[31,32] Based on these observations, we hypothesized that the use of PaX_{560} as a ligand for QacR would enable photo-regulation of the QacR activity for controlling the transcription of target genes. Moreover, we developed a P_{T7}/QacR promoter system by integrating this QacR–PaX_{560} module into the T7 RNA polymerase (T7RNAP) expression system to demonstrate that optical control of gene expression is feasible both in a cell-free system and live bacterial cells.

Results and Discussion

The working of our QacR–PaX_{560} system relies on binding of a cationic lipophilic dye to the transcription factor QacR and positive-charge acquisition of the dye upon photo-irradiation. Structural analysis of the QacR–ligand complex revealed that binding of cationic compounds is stabilized by hydrophobic and anionic amino acid residues in the ligand binding pocket through π–π, π-cation, and ionic interactions.[31] In addition, the large volume of the ligand-binding pocket of QacR allows flexibility in the structure of ligands it can bind. Hence, we reasoned that PaX_{560}-CF, the cationic form of PaX_{560}, could serve as a QacR inducer, albeit an artificial ligand. Before light irradiation, QacR is thought to bind to the operator sequence IR1 and inhibit RNA polymerase (RNAP) from contacting the promoter region, thereby inhibiting mRNA synthesis (Figure 1). Since QacR only recognizes cationic lipophilic compounds, transcription remained suppressed after the addition of the neutral PaX_{560}. However, once the illumination of blue light (405 nm) is applied, PaX_{560} becomes converted to positively charged PaX_{560}-CF. The charge transition leads to increased affinity for QacR, forming the QacR/PaX_{560}-CF complex. This entails the detachment of QacR from the IR1 sequence, which results in the initiation of transcription by RNA polymerase.

Amongst several PaX_{560} derivatives, we selected the derivative with an α-methyl vinyl substituent (Figure 1), as it exhibits the fastest photoactivation.[26] PaX_{560} and PaX_{560}-CF were synthesized according to the published method.[26] As reported, an increase in the absorbance at 560 nm was observed after irradiation of 3 mW/cm^2, indicating the conversion into the fluorescent form (Supporting Figure S1). The absorbance measurement at 560 nm revealed that the photoconversion is completed by mild irradiation of 3 mW/cm^2 at 405 nm for 60 sec (Supporting Figure S2). Since cationic PaX_{560}-CF shows fluorescence, the dissociation constant towards QacR was determined by fluorescent polarization assay. Titration of QacR into 50 nM of PaX_{560}-CF yielded a K_d of 0.87 μM (Supporting Figure S3), which is in good agreement with the dissociation constants of reported ligands, such as Rhodamine 6G (0.20 μM), pyronine Y (5.15 μM), and ethidium bromide (0.81 μM).[34] This sub-micromolar K_d value is clear evidence that PaX_{560}-CF displays a strong affinity towards QacR. Further, to verify that PaX_{560}-CF shows a stronger affinity towards QacR than neutral and non-fluorescent PaX_{560}, we measured the dissociation constants for those compounds by isothermal titration calorimetry (ITC). Titration of QacR with increasing concentrations of PaX_{560} led to no change in heat (Figure 2, left panel). On the other hand, titration with PaX_{560}-CF under the identical condition yielded a dissociation constant of 3.0 μM (Figure 2, right panel). This result confirmed that cationic PaX_{560}-CF has a significantly higher affinity to QacR compared to neutral PaX_{560}, making it an ideal light-controllable effector for the transcription factor.

To utilize *Escherichia coli* BL21 (DE3) as a host strain for the light-controlled QacR–PaX_{560} transcription system, we newly developed a P_{T7}/QacR promoter system that allows transcriptional initiation of T7RNAP to be regulated by QacR. QacR recognizes the operator sequence IR1 located directly adjacent to a promoter sequence, thereby preventing the transition of the RNAP-promoter complex into a transcriptionally productive state.[31] The sequence of IR1 and the docking site for QacR have been fully identified by DNase I footprinting assay[32] and QacR–DNA complex crystal structure analysis,[33] respectively. The crystal structure has provided us with an insight that 24-bp sequence 5′-TATA GACC GATC GCAC GGTC TATA-3′ is the minimum IR1 (IR1 mini) bases required for QacR recog-

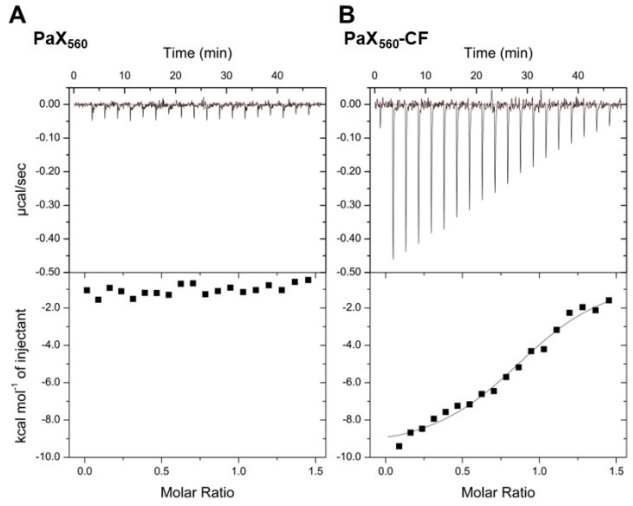

Figure 2. Comparison of affinity for QacR between PaX_{560} and PaX_{560}-CF determined by ITC. Titration of QacR with the two ligands (A) PaX_{560} and (B) PaX_{560}-CF were implemented under identical condition, where the concentration of QacR was 30 µM and that of ligands was 225 µM. Data were analyzed using a single-site-binding model.

nition. We thought to position IR1 mini downstream of the P_{T7} sequence to block T7RNAP from docking to the promoter sequence. Inspired by the work by Dubendorfft and Studier on developing the P_{T7}/LacI promoter system,[33] we prepared a set of DNA constructs with varying numbers of bases between the first base transcribed in P_{T7} (guanine) and the first base in IR1 mini (thymine) to explore the optimum sequence that can minimize basal transcription (Figure 3A). To determine the optimal construct, in vitro T7 transcription assay was used. Transcription reaction was carried out for 1 h at 37 °C using a thermal cycler, and RNA production was quantified by band intensities on denaturing polyacrylamide gel stained with ethidium bromide (Supporting Figure S4 and Supporting Table S3). The result indicated that transcription inhibition by QacR was enhanced with decreasing number of bases between P_{T7} and IR1 mini (Figure 3B). Indeed, the RNA production level using Seq 1 as a template was reduced to 2 % of the reaction in which QacR was omitted. Shorter distance between P_{T7} and IR1 mini appears to allow QacR to block T7RNAP from binding to the promoter sequence more efficiently. Previous DNase I footprinting experiments have reported that DNase could not access 2 bp each from the terminal nucleotides of IR1 mini in the presence of QacR, suggesting that QacR prevents T7RNAP from contacting the DNA sequence up to 2 bp from IR1 mini.[28] Seq 1, where IR1 mini is juxtaposed to P_{T7}, displayed the maximum transcription inhibition, and this result corresponds well with the previous report on the P_{T7}/LacI promoter system.[33] Therefore, Seq 1 was employed in our P_{T7}/QacR promoter system for further experiments.

Next, we evaluated whether the light-mediated conversion of PaX_{560} to positively charged PaX_{560}-CF can inhibit the transcriptional repression activity of QacR in the in vitro T7 transcription assay. Various concentrations of PaX_{560} (0, 5, 10, 15, 20, and 25 µM) were added to the transcription reaction mixture, and the samples were photoirradiated at 405 nm at 3 mW/cm^2 for 60 sec. The relative band intensities of the transcribed RNA on denaturing polyacrylamide gel were determined by comparing it to non-irradiated controls. RNA production intensified in proportion to the concentration of PaX_{560} for the light-irradiated sample, while it remained relatively unchanged for the non-irradiated controls (Figure 3C, Supporting Figure S5, and Supporting Table S4). In particular, the use of 25 µM of PaX_{560} led to the highest induction (23-fold) relative to the control. Furthermore, the levels of transcription achieved upon light activation of PaX_{560} at concentrations over 20 µM approached nearly 100 % of the level observed in the absence of QacR (Figure 3C and Supporting Figure S5). The results indicated that photoactivated PaX_{560}-CF induced detachment of QacR from IR1 to permit RNA synthesis by T7RNAP. Taken together, the series of experiments described above served as the proof-of-concept for the PaX_{560}-based light-controlled P_{T7}/QacR promoter system we designed.

Although QacR has been extensively characterized biochemically and structurally,[34,35] its utility in the cellular protein expression system has remained limited in part due to the infectious nature of the host bacteria, *S. aureus*. Thus, to investigate the operability of the system in live cells, we proceeded to install it in *E.coli* BL21-(DE3), one of the most commonly used bacterial strains for recombinant protein production in the field of biotechnology[36] that carries a copy of the T7 RNAP gene on its chromosome.[37] To introduce the system into the host, we constructed a plasmid pUC19-QacR-LuxCDABE containing a *luxCDABE* operon[38] under the control of the P_{T7}/QacR promoter system and a QacR gene encoded in the direction opposite to the *luxCDABE* operon under the control of a P_{lac}/lacI promoter system (Figure 4A). With this system, we performed real-time monitoring of the luciferase expression level by measuring the luminescence intensity of the bacterial culture. As anticipated, the addition of PaX_{560} with light irradiation (405 nm, 3 mW/cm^2, 60 sec) resulted in a robust luciferase activity (Figure 4B, left panel). In contrast, the non-photoirradiated control did not show a significant increase in the luciferase activity (Figure 4B, right panel). The luciferase activity level reached the maximum approximately 300 min after light irradiation. At 50 µM PaX_{560}, the induction rate was 42-fold higher relative to the non-illuminated control. This result verified that the P_{T7}/QacR system can activate gene expression in live bacterial cells upon photoactivation of PaX_{560} with low-intensity visible light illumination. The luminescence intensity was increased with the light exposure time (Supporting Figure S6), indicating the amount of luciferase expression is able to be controlled by the extent of light irradiation. With the increasing concentration of PaX_{560}, stagnant bacterial growth was observed among the photoirradiated samples (Supporting Figure S7). This could be due to the influence of the cationic PaX_{560}-CF. In fact, when the microbial viability was examined, while the neutral PaX_{560} exhibited no

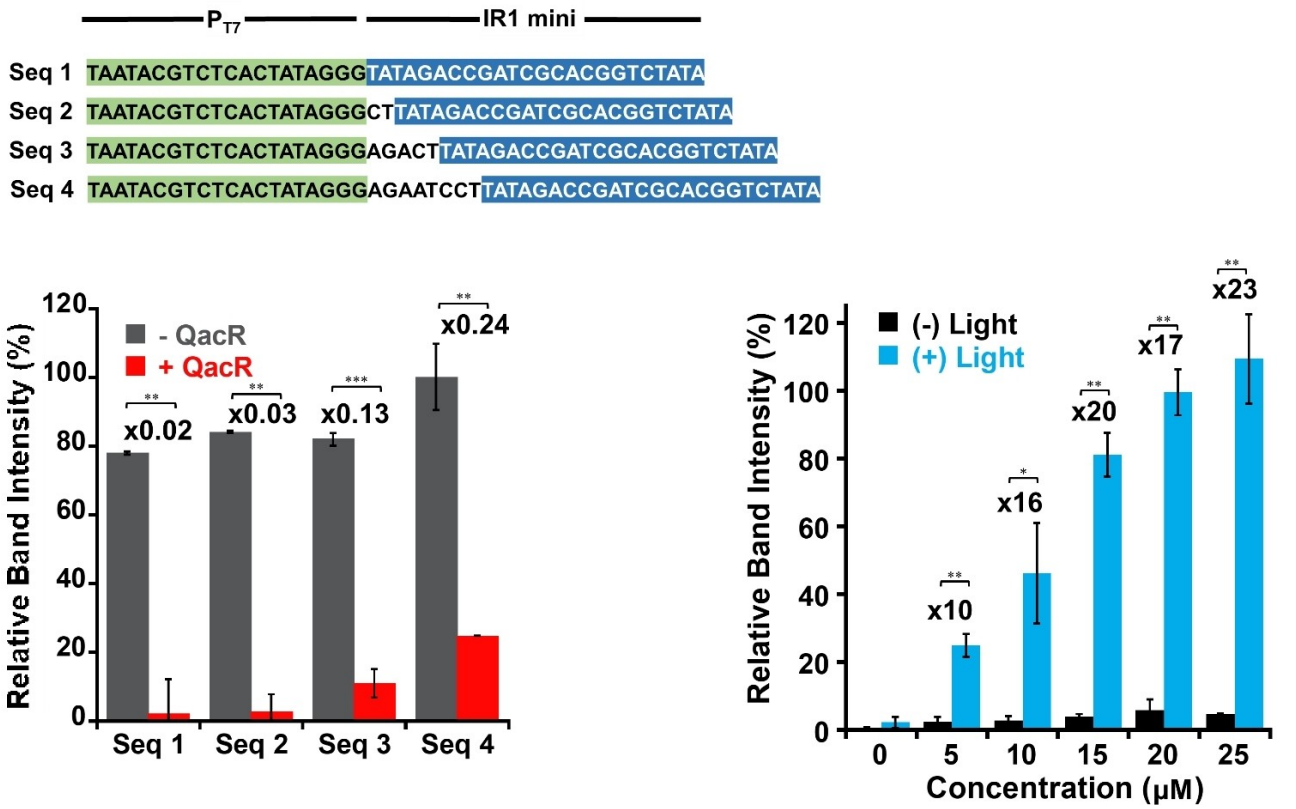

Figure 3. Optical control of in vitro T7 *cell-free* transcription. A) Four constructs Seq 1–4 were examined for optimization of the number of spacer bases between the P_{T7} and IR1 sequences. B) Transcriptional repression by QacR in vitro. QacR (0 or 800 nM) was added to the transcription reaction mixture containing each of the four constructs Seq 1–4 (100 nM) and T7RNAP, and the mixture was incubated at 37 °C for 1 h. The transcribed RNA was quantified by the band intensity on denaturing polyacrylamide gel stained by ethidium bromide (Supporting Figure 4 and Supporting Table 3). C) Photoactivated in vitro transcription. To the transcription reaction mixtures using the Seq 1 construct, PaX_{560} (0–25 μM) was added. Photoirradiation (405 nm, 3 mW/cm^2 for 60 sec) was applied only to the (+) Light samples, and all samples were incubated at 37 °C for 1 h. The RNA was quantified by the same method as described above (Supporting Figure 5 and Supporting Table 4). Error bars denote standard deviation ($N = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significance was evaluated using the Student *t*-test.

toxicity, PaX_{560}-CF affected the growth rate at higher concentrations (Supporting Figure S8).

The distinct advantage of our system is that the activated ligand, PaX_{560}-CF, shows strong fluorescence. Indeed, the fluorescence imaging data showed the photoactivated fluorescence signals of PaX_{560}-CF from the bacteria after photoirradiation, indicating that the dye was internalized into the cytoplasm of the bacteria (Supporting Figure S9). We also found the fluorescence signals from PaX_{560}-treated *E. coli* cells after washing and subsequent light irradiation (Supporting Figure S10), indicating that non-activated PaX_{560} is membrane-permeable and can be taken up by bacterial cells. Hence, this property of PaX_{560} allows simultaneous detection of the state of the effector molecule and recording of the light irradiation event (Figure 4D). To demonstrate this, we used super-folder green fluorescent protein (sfGFP)[39] as a reporter protein to confirm co-localization of PaX_{560}-CF and sfGFP within the cytoplasm by confocal fluorescence imaging. Using pUC19-QacR-LuxCDABE as the parent plasmid, the luciferase operon was replaced with the sfGFP gene to yield pUC19-QacR-sfGFP. Photoirradiation on the pUC19-QacR-sfGFP-transformed *E. coli* BL21(DE3) cell suspension led to activation of PaX_{560}, showing red fluorescence along with enhanced expression of sfGFP after 6 hours (Figure 4E). By contrast, cells without light illumination exhibited neither red fluorescence nor green fluorescence (Figure 4E). In the absence of photoirradiation, the photoactivatable dye remained non-fluorescent and incapable of alleviating transcriptional suppression by QacR, resulting in no induction of sfGFP production. The clear difference in the fluorescence intensities between with and without light irradiation ensures that transcription from our P_{T7}/QacR system is only activated in *E. coli* cells that are showing red fluorescence. Thus, it is safe to conclude that the fluorescence signal from PaX_{560}-CF is an important indicator that allows us to identify the activity state of the effector molecule.

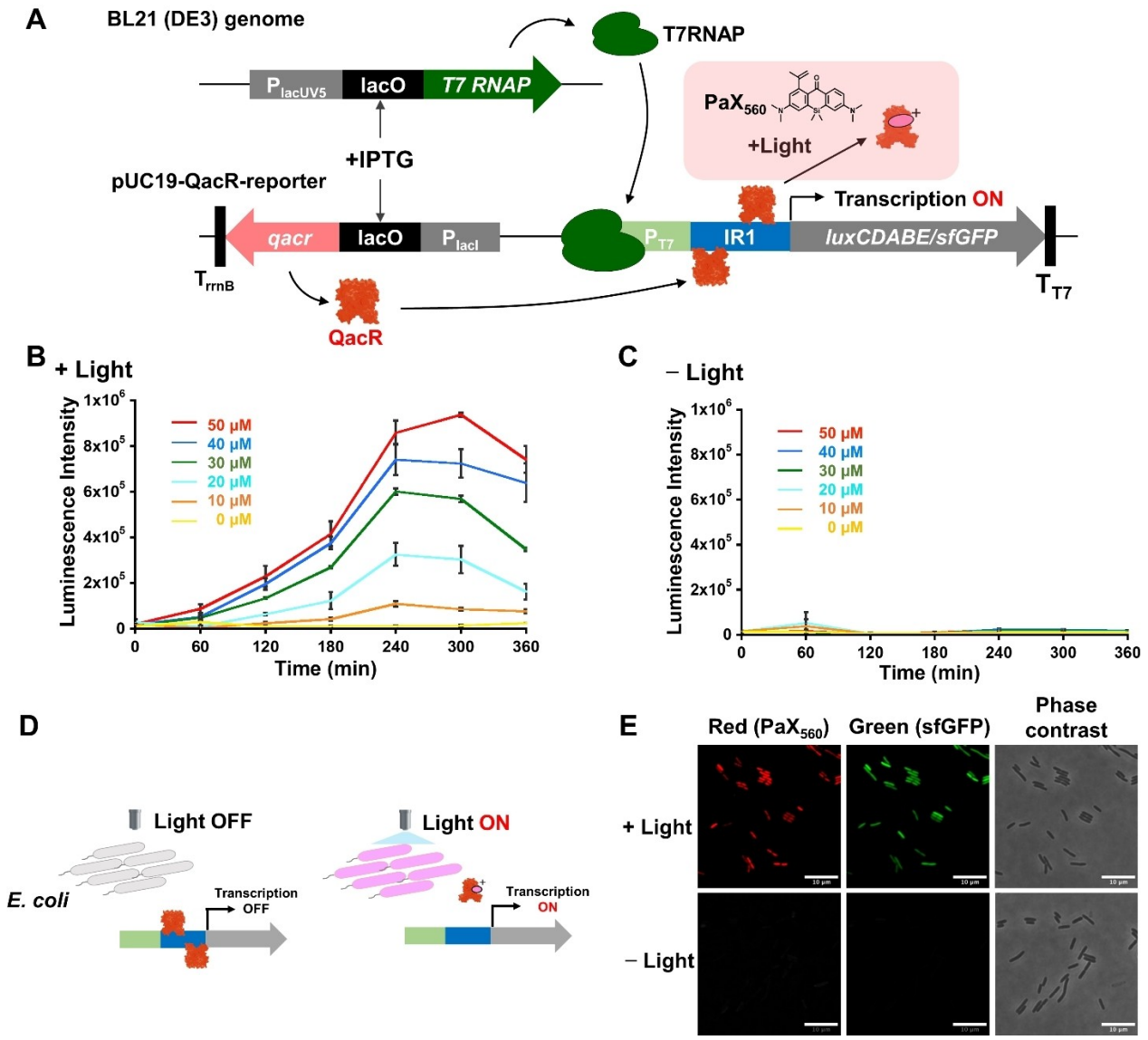

Figure 4. Optical control of T7 transcription in bacterial cells. A) Schematic of the construct used for the luciferase reporter gene assay. T_{rrnB}: *rrnB* transcription terminator; *qacr*: QacR gene; lacO: lac operator; P_{lacUV5}: *lacUV5* promoter; P_{lacI}: *lacI* promoter; P_{T7}: T7 promoter; IR1: IR1 operator; *luxCDABE*: luciferase operon; *sfGFP*: super-folder green fluorescent protein gene; T_{T7}: T7 terminator. B) Light-activated luciferase expression in *E. coli* BL21(DE3) with light irradiation (405 nm, 3 mW/cm^2, 60 sec) and C) without light irradiation. PaX_{560} (0–50 μM) and 0.1% triton X-100 were added to the cell suspension. The cell suspension was photo-irradiated, then incubated at 37 °C with shaking at 900 rpm for 6 h. Luminescence was recorded at the indicated time points. D) Fluorescence from the activated PaX_{560} molecules (pink) provides the memory of photoirradiation. E) Fluorescence imaging of photoactivation of PaX_{560} and sfGFP expression with (top row) and without (bottom row) light irradiation (405 nm, 3 mW/cm^2, 60 sec) in *E. coli*. Images were recorded 6 h after light irradiation. PaX_{560} 50 μM, scale bar: 10 μm.

Conclusion

We have developed a novel class of photochemical transcriptional regulation system using a caging-group-free photoactivatable dye, PaX_{560}, as an effector molecule and QacR as a transcriptional regulator. The key driver of this system is the light-induced charge transition from neutral PaX_{560} to positively charged PaX_{560}-CF. QacR selectively recognizes the cationic form of the dye and loses its repressor function. Thus, transcription is activated upon light irradiation of PaX_{560}. This system is advantageous over the conventional transcriptional regulation system using caged compounds, as its activation neither requires intense UV light irradiation nor generates side products that could interfere with molecular machineries of living organisms. Further, since PaX_{560}-CF is fluorescent but not PaX_{560}, the transcriptionally active effector molecules can be easily identified. The proof-of-concept for our newly developed P_{T7}/QacR promoter system was demonstrated through in vitro transcription assay that showed that light irradiation enhanced RNA production with a maximum of 23-fold. Incorporation of the luciferase reporter genes into *E. coli* BL21(DE3) proved that the system maintained functionality in bacterial cells. Finally, fluorescence

imaging showed that the fluorescence of PaX_{560}-CF could serve as an indicator of the activity state of the transcription factor ligand. The system reported here created a new modality in optical transcriptional regulation that has broad applications in biomedicine, systems biology, and functional genomics. In particular, the use of the popular T7 promoter system should allow our module to be incorporated seamlessly not only into existing in vivo gene expression technologies but also into the field of cell-free systems. Spatiotemporal activation of transcriptional/translational systems encapsulated in liposomes allows remote-controlled production and delivery of gene products from synthetic cells.[21] As such, the reported system could serve as a powerful BioBrick component for developing artificial genetic circuits not only in cell-free systems but also in live bacterial cells.

Supporting Information

The experimental procedures, the synthesis and compound characterization data, spectral data, and supportive images are shown in Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: optical gene expression · transcriptional regulation · photoactivatable dye · multidrug-binding transcriptional regulator · cell-free transcription

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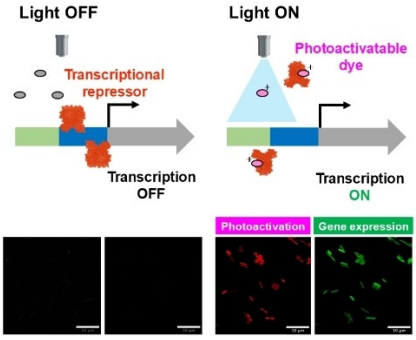
Research Article

Optogenetics

T. Nonomura, M. Minoshima,*
K. Kikuchi* ——— **e202416420**

Light-Activated Gene Expression System Using a Caging-Group-Free Photoactivatable Dye



A light-induced gene expression system was developed using a caging-group-free photoactivatable dye with a multidrug-binding transcriptional regulator, QacR. This system allowed transcriptional activation with mild visible light exposure, large dynamic range, and simultaneous detection of the state of the effector molecule in cell-free and cellular conditions.