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Photocaged T7 RNA Polymerase for the Light Activation of Transcription and Gene Function in Pro- and Eukaryotic Cells

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Abstract

A light-activatable bacteriophage T7 RNA polymerase (T7RNAP) has been generated through the site-specific introduction of a photocaged tyrosine residue at the crucial position Tyr639 within the active site of the enzyme. The photocaged tyrosine disrupts polymerase activity by blocking the incoming nucleotide from reaching the active site of the enzyme. However, a brief irradiation with nonphototoxic UV light of 365 nm removes the *ortho*-nitrobenzyl caging group from Tyr639 and restores the RNA polymerase activity of T7RNAP. The complete orthogonality of T7RNAP to all endogenous RNA polymerases in pro- and eukaryotic systems allowed for the photochemical activation of gene expression in bacterial and mammalian cells. Specifically, *E. coli* cells were engineered to produce photocaged T7RNAP in the presence of a GFP reporter gene under the control of a T7 promoter. UV irradiation of these cells led to the spatiotemporal activation of GFP expression. In an analogous fashion, caged T7RNAP was transfected into human embryonic kidney (HEK293T) cells. Irradiation with UV light led to the activation of T7RNAP, thereby inducing RNA polymerization and expression of a luciferase reporter gene in tissue culture. The ability to achieve spatiotemporal regulation of orthogonal RNA synthesis enables the precise dissection and manipulation of a wide range of cellular events, including gene function.

Keywords

amino acids; caged proteins; light activation; polymerases; RNA

Introduction

The spatial and temporal regulation of biological macromolecules in various cellular processes is vital to developmental progress and cellular differentiation in multicellular organisms. Many of these processes are mediated by the spatiotemporal regulation of RNA production and localization. External rewiring of the cellular circuitry has been achieved by introducing into bacterial and mammalian cells orthogonal RNA polymerases such as bacteriophage T7 RNA polymerase (T7RNAP), which is homologous to the Pol I family of DNA polymerases.^[1]

Photochemical control over protein expression provides a very precise means to study and manipulate gene function with high spatiotemporal resolution.^[2] A biologically active molecule can be rendered responsive to light by installing a light-removable protecting

group (a so-called “caging group”) at a site required for biological activity. Classical caging groups are based on an *ortho*-nitrobenzyl (ONB) framework,^[3] and have been used to photochemically control small-molecule, oligonucleotide, and peptide function.^[2] ONB groups are typically installed on relatively acidic moieties, for example, carboxy and hydroxyl groups, and are removed with nonphotodamaging UV light of 365 nm. Recently, we employed ONB groups in the photochemical regulation of diverse cellular function, including gene expression, gene silencing, ribozyme cleavage, and DNA recombination.^[4, 5]

Rendering T7 RNA polymerase inactive through the installation of a caging group at a defined site will enable photochemical control of gene function, as shown in Figure 1. The orthogonality of the T7RNAP system to all endogenous pro- and eukaryotic RNA polymerases enables the precise light activation of specific genes without interfering with the natural biosynthetic machinery.

T7 RNA polymerase features architectural domains common to the Pol I family of polymerases, specifically a similar right-hand arrangement.^[1] Transcription is carried out by a series of major conformational changes through binding, elongation, and termination stages. In the elongation stage, the mechanism of NTP incorporation follows a four-state catalytic cycle that oscillates between an open and a closed conformation defined by a key domain called the O-helix.^[6] The RNA–DNA complex is moved into the product site of the open complex (Figure 2A).^[7] The subsequent insertion of a matching NTP induces a closed complex formation (Figure 2B).^[8] The phosphoryl transfer is then rapidly catalyzed leading to a relaxation of the enzyme–product complex to the open conformation and allowing the discharge of PP_i and translocation of the template strand.^[9] At the end of the cycle, the polymerase–DNA complex is ready for the next round of NTP incorporation. During the critical open–closed conformational change, Tyr639 in the O-helix motif is believed to play two crucial roles. First, the tyrosyl-OH coordinates Mg²⁺ to interact with the 2′-OH of the incoming NTP to assure discrimination of ribose vs. deoxyribose substrates.^[8] Second, during the translocation process, Tyr639 drives the newly synthesized RNA out of the catalytic site and prevents the RNA–DNA duplex from sliding back until the next NTP is ready for insertion.^[10] Mutational analysis revealed that Tyr639 is critical in this step, since most amino acid replacements resulted in a loss of function or reduced enzymatic activity.^[11]

Recently, we have demonstrated that modification of the corresponding O-helix tyrosine in the homologous family I Taq DNA polymerase with a light-removable ONB protecting group resulted in a completely inactive enzyme.^[12] Irradiation of the caged Taq DNA polymerase with UV light of 365 nm for 5 minutes restored 67 % of activity compared to the wild-type enzyme. Accordingly, we hypothesized that introduction of the caged tyrosine **1**, bearing an ONB group, at the Tyr639 position of T7 RNA polymerase would inactivate it by occupying the space reserved for the incoming NTP with the sterically demanding ONB group (Figure 2C and D). In turn, removal of the ONB caging group through brief irradiation with UV light would generate wild-type T7 RNA polymerase, thus restoring RNA polymerization activity. In contrast to the caged DNA polymerase, a light-activated RNA polymerase has the potential to allow for precise spatiotemporal control of gene transcription (and coupled translation) both *in vitro* and *in vivo*. Due to the orthogonality of the bacteriophage T7 RNA polymerase in pro- and eukaryotic systems, a light-activated version could be used to photochemically reprogram gene function and cellular behavior.

Results and Discussion

Site-specific incorporation of caged tyrosine

In order to incorporate caged tyrosine **1** site-specifically into T7 RNA polymerase at position Tyr639, the previously described in vivo unnatural amino acid mutagenesis approach was employed.^[5,13] The amber stop codon TAG was introduced into plasmid pBH161,^[14] which harbors a hexahistidine-tagged T7 RNA polymerase. The mutated plasmid pBH161Y639TAG was cotransformed with the plasmid pSUPONBY. Protein was expressed with or without **1**, and compared to the expression of wild-type T7 RNA polymerase as a positive control as shown in Figure 3. No protein was visible in the absence of **1**. In the presence of **1**, a protein band corresponding to the wild-type RNA polymerase was observed in a Coomassie Blue-stained SDS-PAGE gel. The site-specific incorporation of **1** and the absence of any natural amino acid at position 639 was verified by mass spectrometry (see the Supporting Information).

Light-activated transcription

We subsequently investigated the light-regulation of RNA polymerase activity in an in vitro transcription assay. Here, wild-type T7 RNA polymerase and mutant Y639ONBY were irradiated in quartz cuvettes with a UV transilluminator (25 W) at 365 nm for 0, 2, 5, 10 and 20 minutes, followed by transcription of an 87 bp DNA template in the presence of α -³²P-ATP. The results were analyzed by urea PAGE and quantified (see Figure 4). In these experiments, no inhibition of wild-type polymerase activity was observed due to UV exposure (data not shown). Product formation was directly proportional to the amount of wild-type polymerase added to the reaction. Most importantly, the caged enzyme was virtually inactive (within the error margin of the quantification method) before UV irradiation, and could not transcribe efficiently. However, it was readily activated by exposure to UV light of 365 nm for 2 min, achieving a maximum of 70 % of wild-type polymerase activity ($2.8 \text{ ng } \mu\text{L}^{-1}$) after 20 minutes' irradiation. Longer irradiation times did not further increase activity. Overall an almost 100-fold increase in RNA production was observed after light activation of the caged T7 RNA polymerase. These experiments confirmed that a light-activatable version of T7 RNA polymerase could be engineered through the site-specific replacement of Tyr639 with the photocaged tyrosine **1**, thus enabling the photochemical control of transcription.

Light-activated coupled transcription translation in vitro

Next, we investigated if the RNA produced by the light-activated mutant T7 RNA polymerase, in comparison to wild-type polymerase, could be successfully translated into functional proteins. The caged and wild-type T7 RNA polymerases were subjected to an in vitro translation system (Promega). As expected, the absence of polymerase led to a low level of luciferase expression. A similar result was obtained for caged T7 RNA polymerase,

thus indicating an almost complete inactivation of the enzyme through the installation of a single *ortho*-nitrobenzyl caging group on Tyr639 (Figure 5). However, a brief irradiation of the caged polymerase for 5, 10, 15, or 20 min at 365 nm, restored expression of the functional luciferase protein; this indicated the high fidelity of the polymerase. In contrast to the in vitro transcription shown in Figure 4, the protein expression levels in the combined transcription/translation assay with caged polymerase displayed a maximum after 10 minutes of UV irradiation (365 nm), and could not be further improved through extended irradiation times (Figure 5). Here, 78 % of wild-type activity was restored through light irradiation.

Light-activated gene expression in bacteria

In order to evaluate the ability to achieve light-activated gene expression through a photocaged T7 RNA polymerase in bacterial cells, pBH161 and mutant plasmids were further engineered to carry a T7-promoter-driven glutathione-S-transferase (GST)-eGFP expression cassette. The plasmids pBH161-T7GST-eGFP and pBH161Y639TAG-T7GSTeGFP were cotransformed with pSUPONBY plasmid into *E. coli* BL21 (Novagen). Addition of isopropyl- β -D-thiogalactopyranoside (IPTG; 0.1 mM) to the *E. coli* BL21 strain harboring pBH161-T7GSTeGFP resulted in T7 RNAP expression, leading to strong eGFP expression. BL21 cells harboring both pSUPONBY and pBH161Y639TAG-T7GSTeGFP yielded low fluorescence, since only truncated, nonfunctional T7 RNA polymerase protein is produced.

In order to demonstrate spatiotemporal control of protein expression, cultures of bacteria transformed with or without pSUPONBY were grown in the presence of **1** (1 mM), harvested, washed with PBS, and immobilized in a low-melt agarose-based matrix. Spatially restricted irradiation from a 365 nm LED fiber optics UV source (Prizmatix) was conducted to produce localized GFP expression within the agarose plate (Figure 6). The irradiated area exhibited a pronounced GFP expression, and the fluorescence strengths of the irradiated locations correlated well with the duration of the UV exposure. These results indicate that caged T7 RNA polymerase could be expressed and photochemically activated in bacterial cells in order to create spatially patterned gene expression.

Light-activated gene expression in mammalian cells

Due to the orthogonality of T7 RNAP to the transcriptional machinery in mammalian cells, we investigated the applicability of light-activated transcription in eukaryotic cells by using the photocaged T7 RNA polymerase. Despite the fundamental differences between eukaryotic and prokaryotic transcription and translation, this polymerase has been successfully employed in gene expression in cell culture and plants.^[15, 16] However, additional plasmid engineering needed to be performed in order to enable translation of T7 RNA polymerase-transcribed mRNA into protein in eukaryotic cells. As a result, the plasmid pT7Luc that encodes a *P. pyralis* (firefly) luciferase under the control of the T7 promoter was modified to contain an internal ribosome entry site (IRES) sequence that was PCR amplified from a pIRES-dsRed plasmid. pT7Luc was used as a template to generate a long PCR product that contained an NcoI site between the T7 promoter and the luciferase coding sequence by using Phusion polymerase, thus enabling IRES sequence insertion. Wild-type and caged T7 RNA polymerases (200 ng) were cotransfected with the pT7-IRES-Luc plasmid (500 ng) into HEK293T cells according to a lipofectamine-based protocol.^[16]

After transfection, cells were either briefly exposed to UV light of 365 nm (2 min, 25 W) or kept in the dark. UV exposure did not alter the confluency and visual appearance of cells compared to a nonirradiated control. The medium was replaced with standard growth medium to allow for T7-driven luciferase expression. After 24 hours, the medium was

removed, and the cells were assayed by using a luciferase assay system. Without T7 RNA polymerase transfection, only a background level of luciferase expression was detected. However, cotransfection of the reporter plasmid and wild-type polymerase led to a significantly increased luminescence after 24 hours of incubation (Figure 7). No effect of UV irradiation (2 min, 365 nm, 25 W) on the luciferase expression levels was detected. In contrast, light exposure of cells transfected with the photocaged T7 RNA polymerase triggered transcription and expression of luciferase in HEK293T cells, while nonirradiated cells only showed a background level of activity (comparable to the negative control). A more than fivefold increase in luciferase activity was detected after irradiation, corresponding to 62 % of wild-type polymerase activity. This light activation is comparable to the previously obtained in vitro data (Figures 4 and 5). Thus, photochemical activation of T7 RNA polymerase, which is orthogonal to mammalian RNA polymerases, was achieved and employed in the photochemical activation of gene expression in eukaryotic cells. This represents a fundamentally new way of activating gene function in mammalian cell culture with light.

Conclusions

The in vivo incorporation of unnatural amino acids has greatly facilitated protein engineering and has enabled the control and investigation of enzymatic mechanisms on a molecular level.^[12, 17] We have developed a light-activated bacteriophage T7 RNA polymerase through the introduction of a photocaged tyrosine residue at the crucial position Tyr639 within the active site. The caged polymerase enzyme is expressed in bacterial cells engineered with an additional cognate tRNA/tRNA synthetase; this enables the production of substantial quantities of this protein. The photocaged tyrosine disrupts polymerase activity by blocking the incoming nucleotide from reaching the active site of the enzyme, as demonstrated by a transcription assay in vitro. However, a brief irradiation with nonphototoxic UV light of 365 nm removes the *ortho*-nitrobenzyl caging group from Tyr639 and restores the RNA polymerase activity.

The complete orthogonality of T7 RNA polymerase to all endogenous RNA polymerases in pro- and eukaryotic systems allowed for the photochemical activation of gene expression in bacterial and mammalian cells. Specifically, we engineered *E. coli* to produce a photocaged T7 RNA polymerase in the presence of a GFP reporter gene under the control of a T7 promoter. UV irradiation of these cells led to the spatiotemporal activation of T7 RNA polymerase and GFP expression. In an analogous fashion, purified caged T7 RNA polymerase was transfected into human embryonic kidney cells containing a luciferase reporter gene under the control of a T7 promoter. Photochemical activation of T7 RNA polymerase activated RNA polymerization and the expression of luciferase in tissue culture. Thus, the developed light-activated T7 RNA polymerase can be employed in the spatial and temporal control of gene function in pro- and eukaryotic cells. Other conceivable applications include time-resolved studies of RNA polymerization and the light-regulated expression of siRNAs.

Experimental Section

Plasmid point mutagenesis and construction

Plasmid pBH161 harboring His₆-tagged T7 RNA polymerase was a gift from Dr. McAllister (University of Medicine and Dentistry of New Jersey). A point mutation was introduced at Tyr639 to convert the TAC codon to a TAG amber stop codon with a QuickChange II kit and the following primers: GTC ATG ACG CTG GCT TAG GGT CCA AAG AGT TCG GC and GC CGA ACT CTT TGG ACC CTA AGC CAG CGT CAT GAC. The new plasmid was named pBH161Y639TAG. Two plasmids were constructed for the in vivo

expression of reporter genes under T7 promoter (Promega) control. The first plasmid, pBH161-T7GSTeGFP (Figure S3 A) was derived from the pBH161 plasmid (Figure S3 B) harboring T7 RNA polymerase under the control of the LacUV5 promoter. The downstream EcoRI site on the backbone was digested to allow the insertion of a T7-promoter-driven reporter cassette. The reporter cassette was PCR amplified from a pET41-based GST-eGFPn1 plasmid (Figure S3 C) with primers containing an EcoRI site (promoter: CGC GAA TTC TAA TAC GAC TCA CTA TAG GG, terminator: CGC GAA TTC ATA GTT CCT CCT TTC AGC). The orientation of the GFP gene was confirmed by PCR screening and DNA sequencing. The second plasmid, T7IRESLuc (Figure S4 A), was constructed by PCR with Phusion polymerase (NEB; Ipswich, MA, USA) and a reverse primer containing a BamHI site (T ATT GGA TCC GGG CCC TCT AGA TGC), a forward primer containing an NcoI site (CA CAA CCA TGG AAG ACG CCA AAA ACA TAA AG), and the backbone plasmid T7Luc (Promega; Figure S4 B). The plasmid pIRES-dsRed (Clontech) was digested with BamHI and NcoI sites, and the resulting 580 bp fragment was purified from an agarose gel. The IRES sequence was then ligated to the long PCR product to generate the IRES-containing fusion gene. The promoter and coding sequence of the IRES-Luciferase gene was sequenced to make sure no mutation was introduced.

Protein expression

The mutated plasmid was cotransformed into the *E. coli* BL21 strain together with the pSUPONBY plasmid, which encodes six copies of the *Methanococcus jannaschii* tRNA^{tyr}_{CUA} and one copy of the evolved *M. jannaschii* tyrosyl-tRNA synthetase gene.^[18] Cells were cultured in the presence of ampicillin (50 mg L⁻¹) and chloramphenicol (34 mg L⁻¹) in LB medium. A solution of ONBY (**1**; 100 mM) in DMSO was prepared freshly and added to the medium with constant stirring to a final concentration of 1 mM. The inoculated culture was incubated at 37°C for 4 h to reach an OD₆₀₀ of 0.6, and IPTG (1 mM final concentration) was added to induce lacUV5-controlled protein expression. Cells were pelleted, lysed, and purified in one step with Ni-NTA agarose beads (Qiagen) according to the manufacturer's protocol, except for the presence of NaCl (600 mM) in the binding and washing buffer. The eluent containing the purified protein was loaded onto a Centricon 50-kDa-cutoff column (Millipore) for a buffer exchange to PBS buffer at pH 7.4. The protein concentration was determined by Bradford assay (Biorad) by using bovine serum albumin (NEB) as a standard. Wild-type protein, caged protein, and cell extract from an expression in the absence of **1** was then loaded onto a 8 % SDS-PAGE gel and submitted to electrophoresis at 150 V for 1 h. The samples could be kept at 4 °C for 2 weeks without apparent loss of enzymatic activity.

Light-activated transcription in vitro

To evaluate the restoration of enzymatic activity after decaging, wild-type or caged T7 RNA polymerase (467 ng) was diluted in Millipore water (100 µL). Decaging was carried out in a quartz cuvette (Fisher Scientific) horizontally placed on a UV transilluminator (UVP). Aliquots (6 µL) were removed from the cuvette after 0, 2, 5, 10, and 20 min of UV irradiation at 365 nm. After decaging, a master mix (4 µL) containing α-³²P ATP (0.25 µL; MP Biomedical, Solon, OH, USA), NTP mix (0.4 µL, 12.5 mM each nucleotide; NEB), 5× T7 RNA polymerase buffer (2 µL; NEB), oligonucleotide template (1 µL, 25 µM), and RNase inhibitor (0.2 µL, 5 units; Promega) was added to decaged RNA polymerase (6 µL). Template sequence: TAA TAC GAC TCA CTA TAG GGC GAC CCT GAT GAG CCT GGA TAC CAG CCG AAA GGC CCT TGG CAG TTA GAC GAA ACG GTG AAA GCC GTA (87 bp, the promoter region is underlined). The reactions were carried out in a thermocycler at 37 °C for 2 h, and the products were separated on a 10 % urea Tris-boric acid-DTA polyacrylamide minigel. Electrophoresis was performed at 150 V for 45 min in a vertical PAGE unit (Biorad). The gel was exposed to a phosphor imaging screen overnight,

and the screen was scanned on a Storm 840 (GE Healthcare) and quantified with ImageQuant 5.2.

Light-activated transcription-coupled translation in vitro

A rabbit reticulocyte lysate provided the translational machinery to produce luciferase from a T7-promoter-driven cassette (pT7Luc, Promega). The T7 RNA polymerase provided in the kit was replaced with wild-type or decaged mutant T7 Y639ONBY RNA polymerase. The decaging conditions were virtually the same as those described in the in vitro transcription section, except that the irradiation times were adjusted to 0, 5, 10, 15, and 20 min. Reactions were conducted with no polymerase (control), recombinantly expressed wild-type polymerase (70 ng per 25 μ L reaction volume), and caged T7 RNA polymerase (70 ng per 25 μ L reaction volume). The transcription/translation was carried out in a thermocycler at 30 °C for 90 min. The activity of the expressed luciferase reporter was measured on a Wallac VICTOR³V luminometer (PerkinElmer) by using a luciferase assay system substrate (Promega). Three independent reactions were carried out for each decaged polymerase solution.

Light-activated gene expression *E. coli*

BL21 cells were cotransformed with pSUPONBY, pBH161-T7GSTeGFP, and pBH161Y639TAG-T7GSTeGFP to allow the detection of decaged T7RNA polymerase activity in vivo. LB medium (20 mL) containing IPTG (0.1 mM) was used to induce caged T7 RNA polymerase production. The culture was inoculated with an overnight culture (80 μ L) with ampicillin (50 mg L⁻¹) and chloramphenicol (34 mg L⁻¹) and incubated for ~4 h to reach an OD₆₀₀ of 0.6. Cells were harvested by centrifugation and resuspended in LB medium containing 1% low-melt agarose, food coloring, and IPTG (0.1 mM) at 37 °C. The medium was then transferred to a 35 mm Petri dish and was allowed to solidify at room temperature for 20 min. The plates were then segmented into six portions and each one received localized UV irradiation for 0, 0.5, 1, 2, 5, or 10 min (365 nm, UV LED fiber optics system, Prizmatix, Southfield, MI, USA).

Light-activated gene expression in mammalian cells

Human embryonic kidney (HEK293T) cells were passaged into two 96-well culture plates, and grown to 60 % confluence. The cells were then transfected with T7IRES-Luc (0.5 μ g) and T7 RNA polymerase (WT or caged, 0.2 μ g) by using Lipofectamine 2000 (1 μ L per well; Invitrogen) in OptiMEM medium (Invitrogen). The transfected cells were incubated at 37 °C for 2 h, after which time the medium was removed and one plate was irradiated at 365 nm (2 min, 25 W, UVP Transilluminator). The medium was replaced with standard growth medium (Dulbecco's modified Eagle's medium with 10 % fetal bovine serum (Hyclone, ThermoFisher, Waltham, MA, USA) and 2 % streptomycin/ampicillin (MP Biomedical)) and incubated for 24 h at 37°C (5 % CO₂) to allow for T7-driven luciferase expression. Experiments under all incubation conditions were conducted in triplicate on each plate. Following a visual inspection, the medium was removed, and the cells were assayed with the Luciferase reporter assay system (Promega) by using a Wallac VICTOR3V luminometer with a measurement time of 1 s and a delay time of 2 s. The firefly luciferase expression calculated for each of the triplicates was averaged, and standard deviations were calculated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Application of a caged, inactive T7RNAP in the photochemical regulation of orthogonal gene expression through UV irradiation.

Figure 2.

A) Model of the T7RNAP polymerase open complex (preinsertion). B) Model of the RNA polymerase closed complex (insertion). C) Hypothesized model of the inactive caged RNA polymerase (ONB = *ortho*-nitrobenzyl). D) Crystal structure of the active site of T7RNAP, indicating the tyrosine Tyr639 and the central magnesium ion (PDB ID: 1S0V). The DNA template is shown in yellow, and the incoming nucleotide analogue diphosphomethyl-phosphonic acid adenosyl ester (APC) is shown in blue. Numbers indicate the length of dashed lines in Å.

Figure 3.

Expression of photocaged T7RNAP Y639ONBY. Lane M: NEB broad-range protein marker. Lane 1: Ni-NTA purified expression from the pBH161-Y639TAG plasmid in the absence of **1**. Lane 2: Ni-NTA purified expression in the presence of **1** (1 mM). Lane 3: Ni-NTA purified expression of wild-type T7RNAP from the pBH161. The yield of photocaged T7RNAP was 0.8 mg L⁻¹ after Ni-NTA purification.

Figure 4.

Light-activated in vitro transcription by the caged T7RNAP depending on the duration of irradiation. Caged T7RNAP (28 ng per 10 μ L reaction volume) was irradiated on a UV transilluminator (25 W) at 365 nm for 0, 2, 5, 10 and 20 min. The transcription reaction was then started by adding a nucleotide substrate master mix (containing α - 32 P-ATP), and the reaction was stopped after 120 min by the addition of 2 $\times$ RNA loading buffer containing urea. The samples were separated on a 10 % urea-polyacrylamide gel, and the gel was exposed to a phosphorimager cassette to be visualized by using a Storm 840 Phosphorimager, and the RNA product was quantified by using ImageQuant 5.2 software. The results were normalized to labeled RNA generated by wild-type T7RNAP at three concentrations (28, 14, and 9 ng per 10 μ L reaction volume) All experiments were conducted in triplicate and normalized to wild-type T7RNAP (28 ng). The error bars represent standard deviations.

Figure 5.

Light-activated in vitro transcription/translation of luciferase. Caged T7RNAP (70 ng per 25 μ L reaction volume) was decaged on a transilluminator (25 W) at 365 nm for 0, 5, 10, 15 and 20 min. In vitro transcription/translation reactions were initiated by adding wild-type and irradiated T7RNAP to a TNT-coupled rabbit reticulocyte system and plasmid pT7Luc (Promega). Luciferase activity was measured with a luciferase assay system on a VictorX5 luminescence plate reader (Perkin-Elmer). Control: no polymerase added. All experiments were conducted in triplicate and normalized to wild-type T7RNAP (70 ng). The error bars represent standard deviations.

Figure 6.

In vivo spatiotemporal activation of transcription in *E. coli* cells. Bacteria were cotransformed with pSUPONBY and pBH161Y639TAG-T7GSTeGFP. Cells were grown in LB medium containing IPTG (0.1 mM), and without (A) or with (B) 1 (1 mM). Cultures were harvested at an OD₆₀₀ of 0.6 by centrifugation and resuspended in LB medium containing 1 % low melt agarose. The suspension was transferred to a 35 mm plate and allowed to solidify. The plates were then segmented into 6 portions and each one of them received point UV irradiation of 365 nm for 0, 0.5, 1, 2, 5, and 10 min (in a clockwise fashion, UV LED fiber optics system, Prizmatix). The plates were then incubated at 37 °C for 16 h and placed in a 4 °C refrigerator for 24 h before imaging with a 365 nm excitation wavelength (Eagle Eye II, Stratagene).

Figure 7.

In vivo light activation of T7RNAP-driven protein expression. HEK293T cells were cotransfected with pT7-IRES-Luc and no polymerase (control), wild-type T7RNAP, or caged T7RNAP. Cells were either kept in the dark (–UV) or irradiated for 2 min at 365 nm (+UV). Luciferase activity was measured after 24 h. All experiments were conducted in triplicate, and the error bars represent standard deviations.