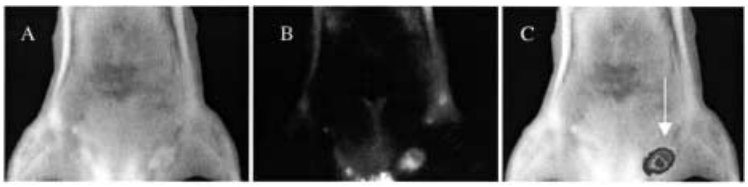

Figure 3. *In vivo image of an OVCAR3 tumor in a nude mouse. The tumor was grown for 9 weeks to reach a size of approximately 3 – 5 mm and groups of animals were injected intravenously with 2 nmole NIR2 – folate conjugate. The images show white light (A) and NIR (B) views and a superimposed false-color image of A and B (C). NIR-fluorescent tumor enhancement was acquired with narrow bandpass filters (excitation: 630 ± 15 nm; emission: 700 ± 20 nm). Note the enhancement of the receptor-positive tumor in the lower right abdomen (arrow).*

imaging agent, no appreciable enhancement could be observed at the 1- or 24-hour time points after injection.

In conclusion, we have shown that near-infrared fluorochromes can be modified by small molecules other than peptides and can be used for targeting receptor systems. These probes, and the modifications thereof, open the door to more extensive biological and medical applications. Apart from use of the conjugates in cell-based assays, it should be feasible to conjugate libraries of small molecules for rapid identification of targeting ligands. The developed compounds may also be used for the detection of small tumors by endoscopy,[1, 19] noninvasive measurement of receptors in vivo,[6] and determination of the efficacy of receptor-targeted therapeutic agents.

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Photoregulation of the Transcription Reaction of T7 RNA Polymerase by Tethering an Azobenzene to the Promoter

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KEYWORDS:

azo compounds · DNA recognition · enzyme catalysis · photochemistry · RNA

Regulation of gene expression by external stimuli is one of the current important and attractive themes.[1] If gene expression could be triggered or terminated only by photoirradiation at a specific wavelength, the scope of application of photoregulation should be extended. For this purpose, we have synthesized photoresponsive DNA that carries an azobenzene moiety in the side chain of a residue and successfully photoregulated the formation and dissociation of its duplex and triplex. The planar *trans*-azobenzene moiety intercalated between the base pairs stabilizes the duplex as a result of stacking interactions, whereas nonplanar *cis*-azobenzene destabilizes the duplex.[2] Furthermore, elongation of primer DNA by T7 DNA polymerase has been successfully photoregulated by use of this photoresponsive DNA as a modulator.[3] Photoregulation of gene expression requires either the transcription or translation step to be controlled by light irradiation. In the present paper, an azobenzene moiety is tethered to the promoter sequence and transcription by RNA polymerase (RNAP) is photoregulated. Here, we use T7 RNAP because useful in vitro translation systems

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coupled with T7 RNAP transcription are already established for protein synthesis.[4] Introduction of an azobenzene moiety at the appropriate position of the promoter sequence allows effective photoregulation of transcription to be achieved by irradiation either with UV or visible light.

All the chemically synthesized template and nontemplate DNA strands that involve the T7 promoter are shown in Scheme 1. In this study, partially double-stranded DNAs were used.[5] An azobenzene moiety on a D-threoninol linker (**X** residue, Scheme 1) was additionally incorporated into the T7 promoter of a nontemplate strand, designated Azo-**n** (**n** = **9** to **13**) in Scheme 1.[2b, 6] [α-^{32}P]ATP was added to the reaction mixture to isotope-label the produced mRNA, which was separated by polyacrylamide gel electrophoresis and analyzed by an imaging analyzer.

When native DNA (**N**; Scheme 1) was used as a promoter for a nontemplate strand, full-length ^{32}P-labeled transcript (17-mer mRNA) was efficiently produced both in the dark and after UV irradiation for 1 min (see Figure 1 A). The transcription rate after UV irradiation (300 nm $< \lambda <$ 400 nm) was almost the same as that under dark conditions, which demonstrates that 1 min irradiation with UV light did not affect the transcription reaction. However, transcription of photoresponsive DNA that included azobenzene between positions -10 and -11 (Azo-**10**) was strongly impeded in the dark (lanes 1 – 3, Figure 1 B, left), with a transcription rate as slow as 1/10 of that with native promoter **N** (closed circles, Figure 1 B, right). Before UV irradiation, almost all the incorporated azobenzene moiety took the *trans* form. Thus, transcription was essentially switched off by *trans*-azobenzene. Irradiation of this reaction mixture with UV light for 1 min at the beginning of incubation caused the *trans*-azobenzene in the promoter to be isomerized to the *cis* form.[7] Transcription proceeded efficiently after this UV irradiation (lanes 4 – 6, Figure 1 B, left) and mRNA was produced more than fourfold faster than under dark conditions (open circles, Figure 1 B, right). The transcription activity of the *cis* form maintained about 60 % that of native promoter **N**. Thus, transcription was efficiently accelerated by *trans* → *cis* isomerization.

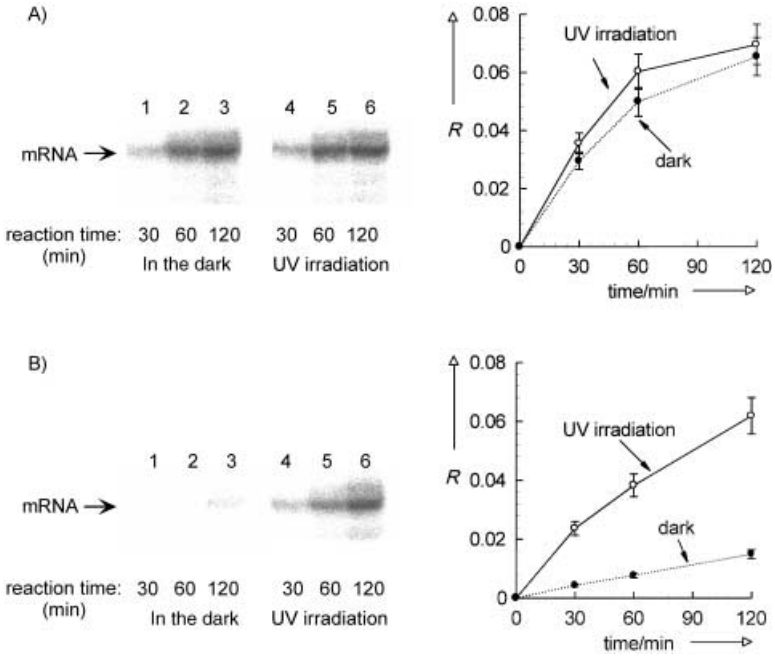
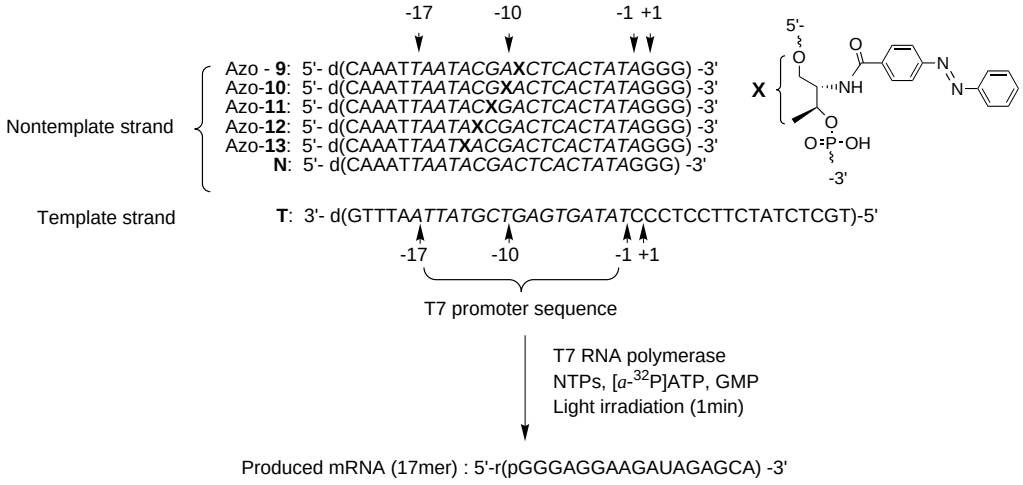


Figure 1. *Time courses of transcription reactions at 37 °C in the dark and after UV irradiation with* ***N*** *(A) and Azo-**10** (B) as nontemplate strands. Left: PAGE patterns of the full-length product. Lanes 1 – 3:* t = *30, 60, 120 min under dark conditions, respectively; lanes 4 – 6:* t = *30, 60, 120 min after UV irradiation, respectively. Right: quantitative plots of the results shown on the left.* R *is defined as the ratio (radioactivity of full-length product)/(total radioactivity of all the oligo- and mononucleotides in the gel). Filled circles: under dark conditions; open circles: after UV irradiation.*

The position of the azobenzene in the promoter significantly affected the photoregulation activity, as shown in Figure 2. Transcription proceeded faster with the *cis* form than the *trans* form of the azobenzene for all **X**-residue positions examined in



Scheme 1. *Sequences of nontemplate (native (**N**) and modified (Azo-**n**)) and template (**T**) DNA strands used in this study, and the experimental procedure for transcription by T7 RNA polymerase. NTP = nucleotide triphosphate; ATP = adenosine triphosphate; GMP = guanosine monophosphate.*

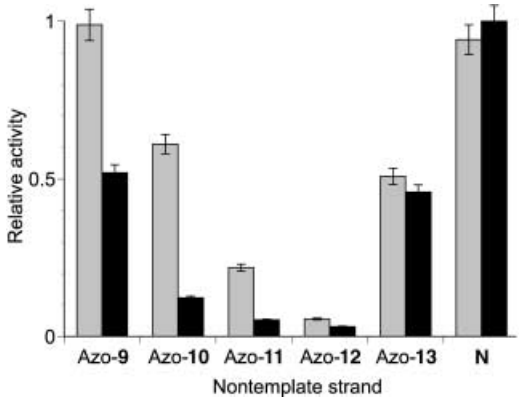


Figure 2. *Effect of the position of an azobenzene moiety on the transcription reaction in the dark (black bars) or with UV irradiation (gray bars). Relative activity is defined as the amount of mRNA produced with respect to that produced with the native promoter* ***N*** *in the dark at 37 °C after 120 min. The reaction mixture was irradiated with UV light for 1 min immediately after incubation at 37 °C began.*

this study.[8] The most effective photoregulation was achieved with Azo-**10** and Azo-**11**; for both these promoters, the increase in the transcription rate caused by *trans* → *cis* isomerization was more than fourfold. Except in these two positions, acceleration of the transcription by isomerization was at most twofold and sometimes much lower.

Transcription by T7 RNAP with Azo-**10**, one of the most effective photoresponsive promoters, as a nontemplate strand, was triggered and terminated with the desired timing either by UV or visible light irradiation, as shown in Figure 3. Transcription was initially switched off in the dark (lanes 1 and 2, Figure 3 A). Upon irradiation with UV light for 1 min (closed arrows, Figure 3 A and B), the "switch" was turned on and transcription started (lanes 3, 4). Transcription was again terminated (lanes 5, 6) by irradiation with visible light ($\lambda > 400$ nm; open arrows, Figure 3 A and 3 B). Transcription switching with the present photoresponsive promoter is clearly demonstrated. In addition to this off-on-off switching, on-off-on switching was successful (see Figure 2 in the Supporting Information).

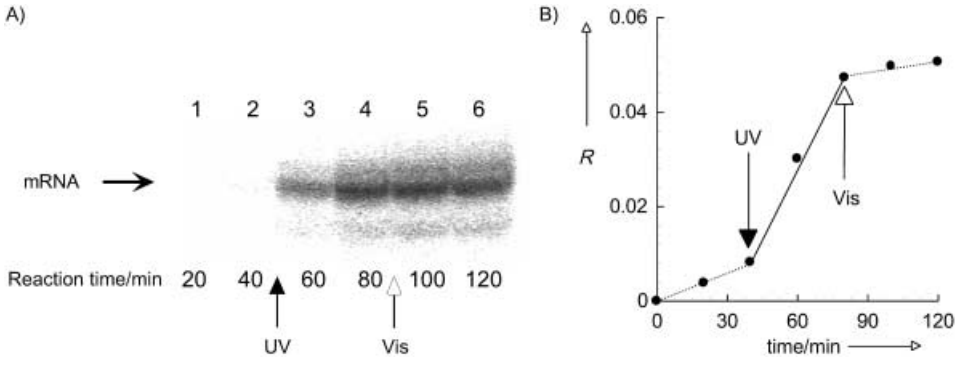


Figure 3. *Off-on-off switching of transcription by irradiation with UV light (filled arrow; switching on) and visible light (open arrow; switching off) at 37 °C with Azo-**10** as the nontemplate strand. PAGE patterns (A) of the full-length product, and quantitative plots (B) are depicted. UV and visible light irradiation was carried out for 1 min at 40 and 80 min after incubation started.* R *is defined as the ratio (radioactivity of full-length product)/(total radioactivity of all the oligo- and mononucleotides in the gel).*

According to Muller et al., footprinting studies of the T7-RNAP – promoter complex with Fe(II)-ethylenediaminetetraacetate·H_2O_2 demonstrated that positions −9 to −14 in the nontemplate strand were protected from hydroxyl radical attack, which indicates that T7 RNAP strongly binds this region.[9] X-ray crystallographic analysis also showed that T7 RNAP recognized the guanosine moiety at the −11 position in the nontemplate strand through water-mediated hydrogen bonding.[10] Presumably, *trans*-azobenzene intercalates between the base pairs and interferes with the binding of T7 RNAP, which strongly suppresses the transcription.[11] The nonplanar structure of *cis*-azobenzene means it cannot intercalate between the base pairs. Rather, it must be flipped out from the helix.[12] As a result, T7 RNAP can bind to the promoter region without significant interruption and transcription proceeds smoothly.

In conclusion, the transcription reaction of T7 RNAP is efficiently photoregulated by use of azobenzene tethered to the T7 promoter.[13] Incorporation of this photoresponsive promoter upstream of the sequence that encodes an important protein is expected to allow in vitro photoregulation of gene expression by T7 RNAP.

Experimental Section

All the photoresponsive DNA sequences (Azo-**n**; Scheme 1) were prepared on an automated DNA synthesizer by using a phosphoramidite monomer that carries an azobenzene.[2b] Transcription by T7 RNAP was carried out according to the literature.[5, 14] The T7 RNAP reactions were performed with T7 RNAP (20 μL, 50 U; from TaKaRa), [α-^{32}P]ATP (20 μL, 2 μCi), each NTP (1 mM), GMP (10 mM), Azo-**n** (or **N**) and **T** (2.0 μM each), and spermidine (2 mM). Tris(hydroxymethyl)-aminomethane(Tris) – HCl buffer (40 mM, pH 8.0) that contained dithiothreitol (5 mM) and $MgCl_2$ (24 mM) was used. First, a mixture of template and nontemplate DNA strands was annealed in Tris – HCl buffer (10 mM, pH 8.0) with NaCl (10 mM) by heating at 95 °C for 3 min and cooling to 37 °C for 30 min. The mixture was then further cooled on ice and a stock solution that contained NTPs, [α-^{32}P]ATP, and GMP was added. After the addition of T7 RNAP, the reaction mixture was incubated at 37 °C for 2 h to achieve transcription. During the reaction, small amounts of the mixture were sampled at desired intervals and transcription was stopped by addition of a dye solution that contained formamide (80 %), EDTA (50 mM), and bromophenol blue (0.025 %) in the same volume as the sampled reaction mixture. This mixture was then subjected to electrophoresis on 20 % polyacrylamide 7 M urea gel. The full-length mRNA (17-mer) separated on the gel was identified by comparison with a chemically synthesized authentic sample and analyzed with an FLA-3000 bio-imaging analyzer (Fuji Photo-Film).

The *trans* → *cis* photoisomerization was achieved by using the UV light from a 6 W UV-A fluorescent lamp (FL6BL-A, Toshiba) to irradiate the sample through a UV-D36C filter (from Asahi Techno-glass) for 1 min. The intensity of the UV light was below 100 μJ s^{-1} cm^{-2}. The

  1439-4227/02/03/08 $ 20.00+.50/0 ChemBioChem **2002**, No. 8

cis → *trans* isomerization was achieved by irradiation with visible light from a Xenon lamp (UV Spot Light Source: HAMAMATSU PHOTONICS) for 1 min through a L-42 filter (from Asahi Technoglass). For the experiments represented in Figure 1 and Figure 2, the solution was UV-irradiated immediately after incubation at 37 °C started. In the experiment represented by Figure 3, UV and visible-light irradiation was carried out when 40 and 80 min, respectively, had passed after incubation started.

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[6] All the modified DNA sequences were characterized by MALDI-TOF MS: found: Azo-**9**, 8030.7; Azo-**10**, 8032.9; Azo-**11**, 8029.9; Azo-**12**, 8029.8; Azo-**13**, 8029.9; calcd for all [Azo-**n** − H]$^+$: 8030.4.

[7] About 25 % azobenzene in the Azo-**10**/**T** duplex was isomerized to the *cis* form under the conditions employed. Note that *trans* → *cis* isomerization by UV irradiation is rather suppressed when the azobenzene is placed in the duplex.

[8] L-Threoninol was also available as a linker for the azobenzene instead of D-threoninol. Although photoregulated transcription was also possible with modified DNA that included an azobenzene tethered on L-threoninol, an azobenzene on D-threoninol was more effective (see Figure 1 in the Supporting Information).

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[11] Since *trans*-azobenzene has a planar structure, it readily intercalates between adjacent base pairs[2a,c]. The intercalated azobenzene should make the duplex re-wind and widen the distance between the neighboring base pairs.

[12] Note that the linker used in this study is rather flexible compared with the natural deoxyribose framework. Therefore, it is possible for *cis*-azobenzene to be positioned in the groove, where it does not disturb the binding of RNAP.

[13] Introduction of multiple azobenzenes or chemical modification of azobenzene is promising for still more effective photoregulation.

[14] We thank Dr. Ichiro Hirao for his useful advice on the experimental protocol of the transcription reaction.

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Site-Directed Mutagenesis of Tyr354 in *Geobacillus stearothermophilus* Alanine Racemase Identifies a Role in Controlling Substrate Specificity and a Possible Role in the Evolution of Antibiotic Resistance

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KEYWORDS:

antibiotics · mutagenesis · protein evolution · racemases · substrate specificity

The pyridoxal 5′-phosphate (PLP) dependent enzyme alanine racemase (EC 5.1.1.1) catalyses the interconversion of the L and D isomers of alanine. In both Gram-negative and Gram-positive bacteria, the D-Ala produced by this enzyme is incorporated into the cell wall as an essential component of the peptidoglycan layer. The structure of the *Geobacillus stearothermophilus*[1] alanine racemase (Alr) has been solved at 1.9 Å resolution,[2] revealing a homodimer in which each subunit consists of two domains: a $(\beta\alpha)_8$ barrel and a C-terminal domain essentially composed of three β sheets (11 strands in total). On dimerisation the $(\beta\alpha)_8$ barrel of one monomer interacts with the C-terminal domain of the other; the active site is a cleft between these two domains. Mutagenic, structural and modelling analyses[3, 4] confirm a catalytic mechanism involving two bases, Lys39 and Tyr265′.[5] The cofactor PLP forms a Schiff base with one of these, Lys39, in the resting enzyme, and in addition to this covalent attachment, a large number of hydrogen bonds fixes PLP rather rigidly in the active site. In particular, the three oxygen atoms of the cofactor phosphate group are involved in an extensive network of hydrogen bonds involving Tyr43, Ser204, Gly221, Ile222 and Tyr354[4] (Figure 1 A). Interestingly however, the structure of an external aldimine form of Alr in which PLP forms a Schiff base with the inhibitor (*R*)-1-aminoethylphosphonic acid (L-Ala-P) suggests that there is ample space in the active site for larger side chains than that of alanine and provides no simple structural explanation for the observed substrate specificity.[4] In the present study we have demonstrated that, rather than merely contributing to the immobilisation of the cofactor phosphate, Tyr354 plays a crucial role in defining the strict specificity of Alr for alanine by preventing turnover of other potential substrates.

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