

form, the steric hindrance of the nonplanar structures may cause the dissociation of part of the duplex so that the RNA is cleaved (Scheme 1 a). Notably, the opening and closing of the big hairpin can be carried out at the single-molecule level. Although several photoresponsive DNAzymes involving one azobenzene unit in the catalytic loop as the photoswitch have been reported, the strategies behind their function are completely different from that here.^[7] Another merit of our design is that the modified DNAzyme should have greater exonuclease resistance than the wild-type one.^[4e]

Figure 1 shows the results of the digestion of an RNA substrate by **Dz7X** at the GU site, which is one of the best substrate sites for the 10–23 DNAzyme.^[6] Here, single-

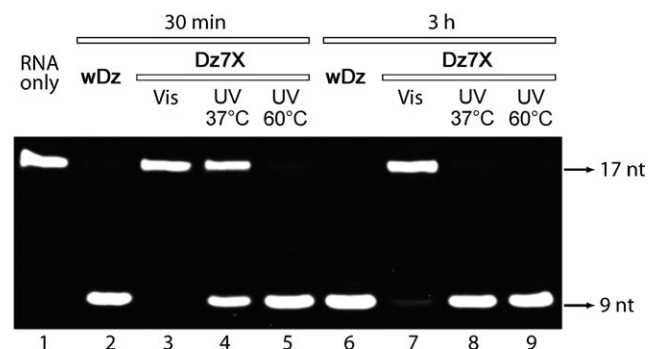


Figure 1. Cleavage of RNA target at the GU site using the photoresponsive DNAzyme **Dz7X**. Lane 1: control (RNA substrate); lanes 2 and 6: positive control of wild-type DNAzyme; lanes 3–5: 30 min of cleavage after irradiation with visible light at 37°C, with UV light at 37°C, and with UV light at 60°C, respectively; lanes 6–9: 3 h of cleavage corresponding to the same samples in lanes 2–5. Conditions: 1.0 μ M RNA, 5.0 μ M DNAzyme, 1.0 M NaCl, 10 mM $MgCl_2$, pH 7.5 (Tris-HCl buffer), at 37°C. The digestion products were analyzed using 20% denatured PAGE electrophoresis.

turnover conditions were used, and the concentration of **Dz7X** was five times higher than that of the RNA substrate. Either UV or visible light was applied before $MgCl_2$ was added to start the reaction. In the case of visible light, the digestion was very slow, and less than 1.8% of RNA was digested after 30 min of reaction at 37°C (lane 3, Figure 1). The melting temperature (T_m) of the stem duplex part involving alternating azobenzene moieties and base pairs was measured to be 72°C, indicating that almost 100% of **Dz7X** was in the hairpin state (see Figure S1 in the Supporting Information). On the other hand, 51.4% of the RNA substrate was digested after 30 min when the DNAzyme had been irradiated with UV light at 37°C for 5 min (lane 4, Figure 1). The acceleration of RNA digestion by irradiation with UV light was as much as 29-fold. After 3 h reaction, only 11.2% of RNA was digested following visible light irradiation, and RNA was almost completely digested in the case of UV light irradiation (lanes 7 and 8, Figure 1). Thus, efficient photoregulation of RNA cleavage was realized by introducing multiple azobenzenes, as outlined in Scheme 1.

Compared with the results obtained with wild-type DNAzyme (**wDz**) (lane 2, Figure 1), the reaction of **Dz7X** after UV light irradiation (at 37°C) showed only about 40%

activity, indicating that not all hairpins were opened under these conditions. UV/Vis spectroscopic analysis showed that about 38% of azobenzene moieties were photoisomerized to the *cis* form when UV light was applied at 37°C (see Figure S2 in the Supporting Information). When UV light irradiation was carried out at 60°C, at which point about 72% of azobenzene moieties had been photoisomerized to the *cis* form and almost all hairpins were open at 37°C, more than 90% activity was recovered compared with **wDz**. This result is reasonable because the RNA/DNAzyme complex in the open state has exactly the same conformation as that of **wDz** (Scheme 1 a). The observed rate constants (k_{obs}) of *trans*-**Dz7X**, *cis*-**Dz7X** (UV at 37°C), and **wDz** were measured to be 0.00071, 0.027, and 0.081 min^{-1} , respectively (see Figure S3 in the Supporting Information for the time-course of RNA cleavage). Thus, the difference in k_{obs} between the reactions with UV and Vis light irradiation was as large as 38-fold.

Similar results were also obtained for cleaving AU, UU, and GC sites (see Figure S4 in the Supporting Information for sequences of DNAzymes and RNA substrates with GC sites), although lower activity was observed (Figure 2). The differ-

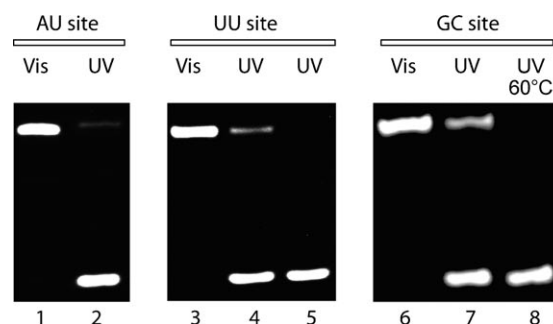


Figure 2. Photoregulation of RNA cleavage at AU, UU, and GC sites using azobenzene-modified DNAzymes. The digestion reaction was carried out for 5 h for the AU site, 18 h for the UU site, and 48 h for the GC site (see Figure S4 in the Supporting Information for sequences). For the UU site, besides **Dz7X** (lane 4), **Dz7X-T** with an additional dT was also used (lane 5). For lane 8, UV light irradiation was carried out at 60°C. Both visible and UV light irradiation were carried out at 37°C for all other experiments. Other conditions are the same as listed in Figure 1.

ence in activity of the DNAzymes between UV and visible light irradiation was 15- to 40-fold. Especially for the UU site, which was barely cleaved by **wDz**, we found that a modified DNAzyme (**DzT**) with an additional deoxythymidine unit (dT) at the junction position between arm II and the catalytic loop could greatly improve activity (lanes 4 and 5, Figure 2). The k_{obs} of **DzT** was 2.7-fold greater than that of **wDz** (data not shown). After UV light irradiation at 37°C a modified DNAzyme with an additional dT unit and seven azobenzene moieties (**Dz7X-T**) displayed an activity even comparable to that of **wDz**. Thus, with **Dz7X-T**, enhancement of activity and clear-cut photoregulation were realized simultaneously for cleaving the UU site. For all the RNA targets examined, the efficiency of the photoregulation was much higher than that achieved with photoresponsive DNAzymes reported previously.^[7] As most RNA-cleaving DNAzymes and ribozymes

use a similar strategy for targeting RNA substrates with two arms, the strategy proposed here can be generalized for constructing other photoresponsive DNAzymes or ribozymes.^[8]

Figure 3 shows real-time photoswitching of RNA digestion at the GU site at 37°C under multiple-turnover conditions. UV and visible light were applied alternatively

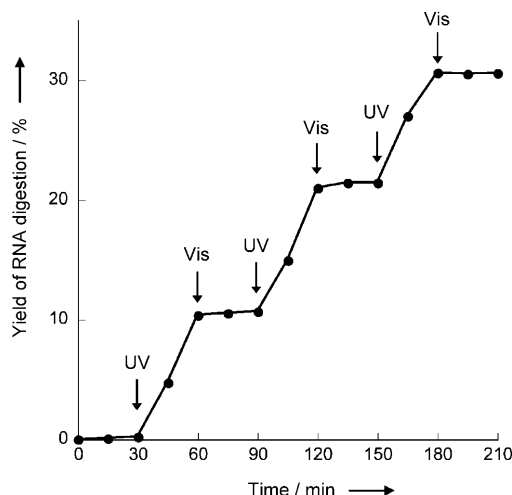


Figure 3. Photoswitching of RNA cleavage at the GU site by **Dz7X**. Visible light was applied at 0, 60, 120, and 180 min. UV light irradiation was carried out at 30, 90, and 150 min. The irradiation time for visible and UV light was 1 and 10 min, respectively.

at a fixed temperature (37°C). The digestion results clearly show that UV light turned on digestion and visible light completely turned it off (Figure 3). Photoregulation was completely reversible and almost no deviation was observed after three repetitions. As the introduced azobenzene moiety is very stable under physiological conditions, this ON–OFF regulation can in principle be repeated many times.^[9] Efficient suppression of RNA digestion by visible light is especially important for in vivo applications, where an RNA transcript can be digested at an exact point and time by control of UV light irradiation.

For evaluating the topological effect on RNA digestion, several DNAzymes with hairpin structures containing a stem 12 base pairs (bp) long and poly(dT) spacers of various lengths were designed (Scheme 1). As shown in Figure 4, when less than two dTs (only one dT in each side) were introduced, for which the topological constraint was much stronger, almost no digestion was observed (see Figure S5 in the Supporting Information for secondary structures). When six dTs were present, cleavage occurred (lanes 3 and 7, Figure 4). When the number of dTs increased to 12, the entire RNA substrate was digested after 4 h (lane 4, Figure 4). Interestingly, even for **nDz-12T**, only partial cleavage activity was recovered compared with that for **wDz**, indicating that the topological constraint influenced the digestion greatly.^[10] This supports our idea that photoregulation of RNA digestion by **Dz7X** occurs according to the strategy shown in Scheme 1. The change of topological conformation of the RNA/DNA-

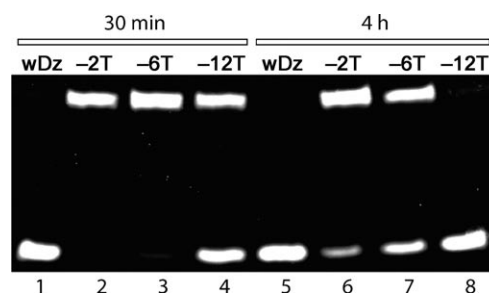


Figure 4. Effect of the topological constraint on RNA digestion by the 10–23 DNA enzyme (see sequences in Scheme 1 b).

zyme complex caused a large difference in cleavage activity between the *trans* and *cis* forms. Thus, we have succeeded in constructing a nanomachine that works on the single-molecule level for photoswitching RNA digestion.

In conclusion, clear-cut and reversible photoregulation of RNA digestion was attained by regulating the higher order structure of DNAzyme/RNA complexes. Our proposed strategy is applicable to other RNA-cleaving DNAzymes and ribozymes that target RNA substrates with two arms, allowing the construction of many photoresponsive DNAzymes or ribozymes. This newly constructed nanomachine is also promising for applications either in vivo for photo-regulating gene expression with an antisense strategy, or in vitro for photoswitching the mechanical movement of a nanorobot.

Experimental Section

Oligonucleotides containing azobenzene residues were supplied by Nihon Techno Service Co., Ltd. (Tsukuba, Japan) and purified by either polyacrylamide gel electrophoresis or HPLC. DNA sequences containing only natural nucleotides were supplied by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The concentration of all oligonucleotides was determined by UV/Vis spectroscopic analysis within 10% error.

Typical procedure for RNA digestion by a DNAzyme: First, a mixture of substrate RNA and the DNA enzyme was annealed in buffer (1 M NaCl, 50 mM Tris-HCl, pH 7.5) by heating at 80°C for 30 s followed by cooling to 37°C for 15 min. Then, either visible light (400–600 nm, 1 min, L-42 filter) or UV light (320–380 nm, 5–10 min, UV-D36C filter, 5.3 mWcm⁻²) irradiation was carried out at 60 or 37°C. The yield of photoisomerization was calculated from the absorbance change at 340 nm (V-530 UV/Vis spectrometer, Jasco, Tokyo, Japan). After this procedure, MgCl₂ was added to start the cleavage reaction, and the reaction mixture was incubated at 37°C. For real-time photoswitching of RNA digestion, UV and visible light were applied alternately after MgCl₂ had been added. During the reaction, a 4 µL aliquot of the reaction mixture was removed and added to 4 µL loading buffer (50 mM EDTA, 45 mM Tris-HCl, 45 mM borate, 7.0 M urea) to terminate the reaction. Finally, 8.0 µL of the resulting mixture was subjected to electrophoresis on a 20% polyacrylamide gel containing 7.0 M urea. Imaging and quantification of the digested RNA were carried out on a Fujifilm FLA-3000G fluorescent analyzer (Fuji Film, Tokyo, Japan).

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