NANO MICRO small

DNA-based nanodevices

Using Gene Regulation to Program DNA-Based Molecular Devices**

*Wendy U. Dittmer, Susanne Kempter, Joachim O. Rädler, and Friedrich C. Simmel**

Molecular machines based on DNA have the ability to perform tasks on the nanometer scale. In addition to movements such as stretching and rotation,[1–8] these devices can execute useful functions such as grabbing and releasing a single protein[9] and walking a defined distance along a circular or linear track.[10–13] As increasingly more machines are developed with functionality, it is desirable to be able to incorporate the devices into living organisms and artificially control within them processes on the molecular scale. Because most DNA nanomachines operate through hybridization of the machine with manually added single-stranded DNA (ssDNA) signals, it is difficult to control the devices in vivo. The integration of the instructions for nanomachine operation into a DNA gene and the genetic regulation of the expression of these instructions can enable these nanodevices to function independently and respond to environmental stimuli. This effort can be viewed in the larger context of using biological design principles for nanotechnology, a field sometimes referred to as synthetic biology.[14–16] In this communication, we demonstrate that the operation of DNA nanomachines can be controlled in vitro using gene regulation switches, in particular those of *E. coli* bacteria.

[*] Dr. W. U. Dittmer, S. Kempter, Prof. Dr. J. O. Rädler, Dr. F. C. Simmel
Department of Physics and Center for Nanoscience, University of Munich
Geschwister Scholl Platz 1, 80539 Munich (Germany)
Fax: (+49) 89-2180-3182
E-mail: simmel@lmu.de

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The expression of genes occurs in two steps: transcription and translation. Transcription of a DNA sequence produces a messenger RNA (mRNA) molecule, which can serve as a template for protein synthesis during translation. Cells can respond to environmental stimuli by the regulation of gene transcription using a variety of mechanisms. One example is the suppression of gene transcription by DNA-binding proteins, which can bind to so-called "operator" sequences and thereby influence the efficiency of binding of RNA polymerase to the "promotor" sequence—the gene sequence where transcription starts—or the efficiency of transcription itself. Small molecules called inducers can also influence transcription by modulating the ability of repressor proteins to bind to the operator sequence.[17]

This ability of cells to alter the expression of their genes in response to environmental changes has been well studied in *E. coli*. The *lac* operon set of genes, which enables the use of lactose in glucose-poor environments, and the SOS regulon genes, required under stress damaging to the cell's genetic integrity, are canonical examples. We used the regulation proteins LexA and LacI of the SOS regulon genes and *lac* operon, respectively, to demonstrate that the instructions for the action of DNA tweezers[1] can be programmed in an artificial gene, and the actuating mRNA signals transcribed depending on the presence of these regulation factors and environmental stimulus. We have shown previously that in vitro generated mRNA can be used to operate nanomachines in the same manner as ssDNA.[18] In the present work, two strategies to turn genes on and off were explored. The first is based on competitive binding to the gene between LexA and RNA polymerase, and the second is based on the physical obstruction of the polymerase by the placement of a LacI obstacle downstream on the gene.

LexA is a regulatory protein that turns off the SOS response under normal conditions.[19,20] It recognizes and binds to a DNA sequence that is highly conserved. An artificial gene was designed that in the 'on' state enabled the transcription of an mRNA signal, which induces the opening of closed tweezers (Figure 1a). An operator sequence for LexA was included upstream that overlapped one base with the promoter sequence of the T7 RNA polymerase so that LexA could hinder polymerase binding. Closed tweezers labeled with a dye and a quencher were incubated with the gene under in vitro transcription conditions consisting of the polymerase, ribonucleotides, and RNAse inhibitor at 33 °C. The operation of tweezers was monitored using fluorescence resonance energy transfer (FRET) experiments. In the absence of the LexA protein, the tweezers automatically open, as indicated by the increase in fluorescence as transcription progresses and mRNA is produced (Figure 1c). However, in the company of LexA the transcription of the signal is deterred and the tweezers open at a slower rate. The half-time for opening the tweezers obtained from an exponential fit to the data increases from 3500 to 5500 s. A larger overlap between the operator for LexA and T7 promoter sequences for complete repression of transcription could not be created without being detrimental to the binding properties of at least one of the proteins. However, by altering the polymerase to a close relative of T7, SP6,[21] it was possible to

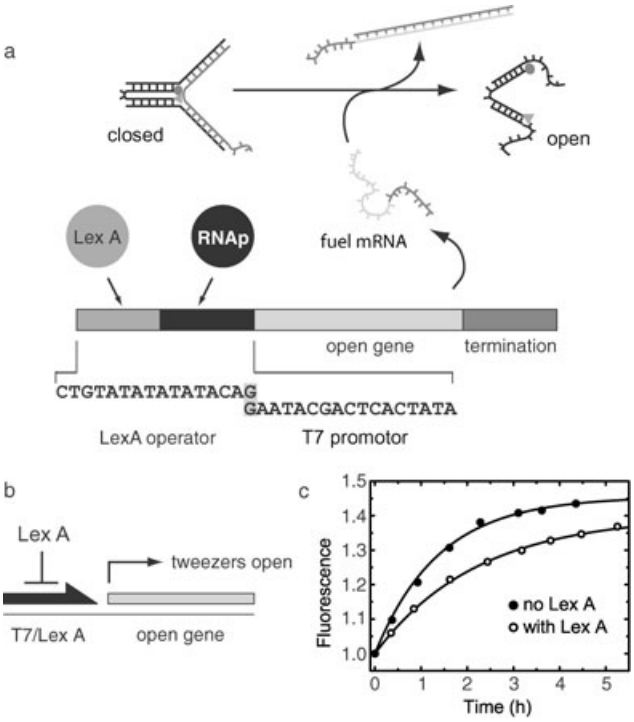

Figure 1. a) Operation scheme and design of the gene with instructions to open closed tweezers by the transcription of an mRNA signal, which is regulated by the competitive binding between LexA protein and T7 polymerase. The promoter and operator sequences were constructed to overlap at one nucleotide base, as highlighted; b) gene circuit diagram for the process; c) FRET experiments monitoring the opening of the tweezers as transcription progresses from the LexA/T7 gene in the absence and in the presence of LexA. Lines are exponential fits to the data. As transcription progresses, fuel mRNA opens the closed tweezers. The tweezers are labeled with a fluorescent dye and a quencher (indicated by a circle and a triangle). The larger distance between these chromophores in the open state leads an increase in the fluorescence signal. This process is slowed down by the presence of the repressor protein.

produce an overlap of two bases by mutating the second base of the promoter sequence from thymine to guanine (Figure 2a). With this modification, the gene is completely turned off in the presence of LexA protein. A polyacrylamide gel electrophoresis (PAGE) of a transcription reaction in the absence of LexA displays bands corresponding to the transcribed mRNA and the gene (Figure 2b). When LexA is present, no mRNA bands are observed and the band corresponding to the gene shifts to form a slower band consisting of the LexA–gene complex. The FRET experiment (Figure 2d) indicates that the opening of the tweezers is fully suppressed in the presence of LexA, and the nanomachine remains in its closed state. In the absence of LexA, the opening reaction progresses more slowly with the SP6 promotor ($t_{1/2}=12000$ s) due to the lower activity of SP6 RNA polymerase.

LacI is a regulatory protein that turns off the *lac* operon genes in the absence of lactose.[22] A gene to instruct open tweezers to close was constructed with a lacI operator sequence placed directly downstream of the T7 promoter sequence (Figure 3a). LacI binds to the operator and hinders the forward progress of the T7 polymerase during transcrip-

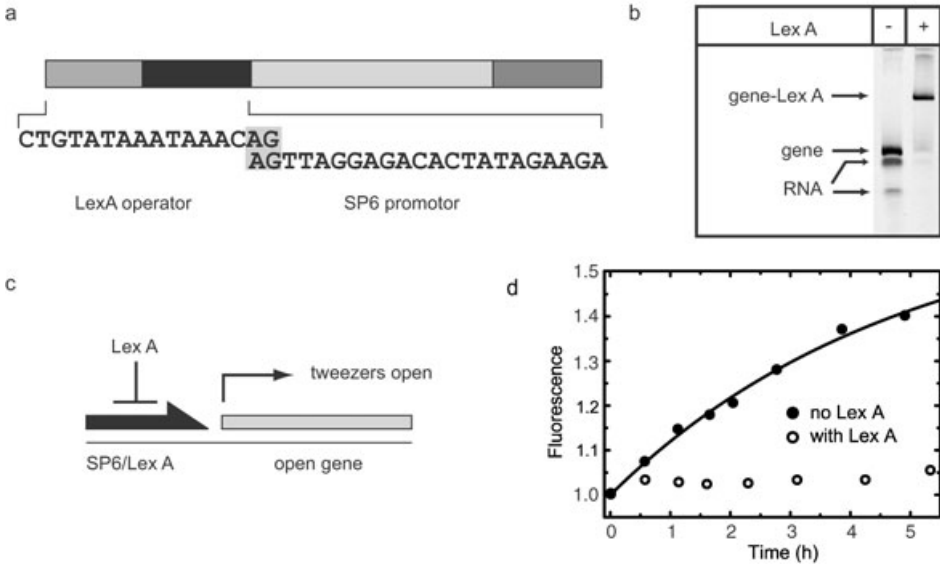

Figure 2. a) A gene similar to that shown in Figure 1a was created with two overlapping bases between the operator for LexA and SP6 promoter; b) a corresponding transcription experiment without tweezers monitored on a 9% PAGE gel. In the absence of LexA, RNA is produced, in the presence of LexA, no RNA is produced and a band for the DNA–protein complex appears; c) corresponding circuit diagram; d) FRET experiments with SP6 polymerase monitoring the opening of the tweezers as transcription progresses from the LexA/SP6 gene in the absence and presence of LexA. LexA completely suppresses the opening of the tweezers.

containing the instructions that is controlled through gene-regulation mechanisms. This was demonstrated for the DNA tweezers system using the gene switches from the SOS regulon and the *lac* operon as examples. Using strategies from the *lac* operon, we were able to couple the response of the tweezers to environmental changes, such as an increase in a lactose analogue. As there are a large number of examples of regulation systems, a multitude of possibilities for programming nanomachines exists. By combining various natural genetic switches capable of interacting with the environment in genetic circuits,[24] it will be possible to program nanomachines and even a group of nanomachines to complete a complex series of tasks and respond accordingly to environmental changes.

tion, resulting in premature termination. Under standard in vitro transcription conditions, the gene without LacI protein allows an mRNA signal to be transcribed, which brings about the closing of the tweezers. The sharp decrease in fluorescence accompanying the closing of the tweezers as mRNA is transcribed was monitored with FRET (Figure 3c). Transcription is repressed with LacI protein. The PAGE experiment shows significantly less-intense mRNA bands when the LacI–gene complex is formed (Figure 3b). Because LacI does not prevent transcription initiation but only extension by T7, the repression is not as effective as with LexA.[23] The complementary FRET experiment with tweezers indicates that LacI significantly hinders the closing of the nanomachines by preventing the expression of the necessary mRNA signal. Additionally, the system is capable of responding to environmental stimulus. In the presence of an analogue of lactose, isopropyl β-D-thiogalactoside (IPTG), transcription is restarted. IPTG is a molecule similar to lactose that binds to LacI and prevents it from attaching to the operator, thus freeing the gene for the polymerase to transcribe full-length mRNA. As seen in the FRET experiment, the environmental change of an increase in the level of lactose analogue causes the system with LacI-regulating proteins to respond by instructing the tweezers to close. The closing rates ($t_{1/2}$ = 3000 s without LacI and $t_{1/2}$ = 5000 s with both LacI and IPTG) are comparable to the opening rates obtained with the T7/open gene. Although the timescales for reactions are compatible for in vivo eukaryotic systems, the mRNA signals produced in prokaryotic systems must be modified to avoid degradation.

In conclusion, we have shown that nanomachines can be made to function independently with the inclusion of a gene

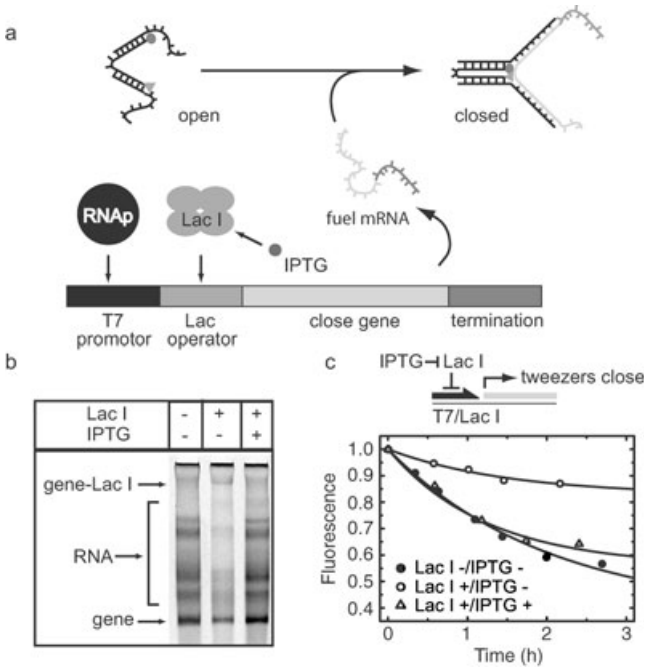

Figure 3. a) Operation Scheme and design of the gene with instructions to close opened tweezers regulated by a LacI switch. The operator sequence is directly downstream of the promoter and LacI blocks transcription extension by T7 polymerase when it binds; b) 9% PAGE of transcription reactions with this gene. The multiple RNA bands are mRNA containing the fuel sequence with different secondary structures and/or lengths; c) FRET experiments monitoring the closing of the tweezers as transcription progresses from the T7/LacI gene in the absence and presence of LacI, and after addition of IPTG.

Experimental Section

DNA sequences: Below is a list of the dsDNA gene sequences used in this work; only the 5′ to 3′ strand is given. The oligonucleotides were custom synthesized and purified with PAGE by biomers.net (Ulm, Germany).

Gene-LacI: 5′ ATGTAATACGACTCACTATAGAGAATTGT GAGCGCTCACAATTTCACGC ATAGACCGTGATTGTTACCAGCGT TAGTTCAGACAGTAGGACTCCTGCTACGAATCCATGATATCTGT TAGTTTTTTTC 3′

Gene-LexA and T7: 5′ AAATTGTGAGCGCTCACAATTCTGTATA TATATACAGAATACGACTCACTATAGACCTCGTAGCAGGAGTCC TACTGTCTGAACTAACGCTGGTAACAATCACGGTCTATGCGATCCAT GATATCTGTTAGTTTTTTTC 3′

Gene-LexA and SP6: 5′ AAATTGTGAGCGCTCACAATTACTGTA TAAATAAACAGTTAGGAGACAC TATAGAAGATCGTAGCAGGAGTCC TACTGTCTGAACTAACGCTGGTAACAATCACGGTCTATGCGCGTTT TATCTGTTTTTTTTTC 3′

Open and closed tweezers were constructed from oligonucleotides described elsewhere.[1] The tweezers were labeled with the dye Oregon Green 488 (Molecular Probes, Eugene, OR) and the quencher BHQ-1 (Biosearch Technologies, Novato, CA) by biomers.net.

In vitro transcription: For a single transcription reaction, dsDNA gene (125 nM), rNTP (rATP, rUTP, rCTP and rGTP from Promega; 5 mM), rGTP (4 mM), $MgCl_2$ (20 mM), dithiothreitol (DTT; 20 mM), bovine serum albumin (BSA; 0.1 mg mL^{-1}), RNAsin (Promega; 150 units ml^{-1}), T7 RNA polymerase (New England Biolabs; 1000 units ml^{-1}), and 1 × RNA polymerase buffer (New England Biolabs) were combined (in that order). Open or closed tweezers were also added at a concentration of 0.4 μM. For reactions with SP6 RNA polymerase the concentration of $MgCl_2$ was lowered to 10 mM due to the sensitivity of the polymerase to salt inhibition. Transcription was completed at 33 °C using T7 and at 37 °C for SP6 as a consequence of the higher efficiency of SP6 at higher temperatures.

LacI protein was synthesized with standard recombinant methods. The *lac* gene was amplified by PCR from the plasmid pET28a(+) (Novagen). The PCR product was inserted into the *Bam*HI and *Eco*RI restriction sites of the plasmid pET23b(+) (Novagen). The pET23b was transformed into *E. coli* strain BL21-(DE3) and induced to express LacI. The recombinant protein contained a 6xHis-tag and was purified using Ni-NTA Superflow (Qiagen) under native conditions. In transcription experiments containing regulation factors, the gene was incubated with the LexA, LacI, or LacI and IPTG for at least 1 hour at room temperature prior to the addition of the other reagents. LacI was added in a ratio of approximately 1:10 dsDNA to the protein in a binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM KCl, 0.1 mg mL^{-1} BSA, 5% glycerol). LexA was added in a ratio of 1:80 dsDNA to the protein. The amount of protein required to achieve complete binding to the DNA was first determined with gel electrophoresis.

FRET experiments: FRET experiments were conducted similarly to those described elsewhere.[1] Fluorescence was excited with an Argon ion laser (488 nm) and detected with photodiodes using standard lock-in techniques. Fluorescence values were recorded for approximately 10 s, approximately every 30 min.

Gel electrophoresis: Non-denaturing PAGE was completed with 9% gels in 1 × TBE (89 mM Tris-borate, 2 mM EDTA) at 10–12 V cm^{-1} for 1 hour at room temperature. To each lane was added approximately 5 μL from a 30 μL transcription reaction, without tweezers. The resulting gel was stained with SYBR Gold (Molecular Probes). Gels to determine protein binding to DNA were completed similarly.

Keywords:

DNA · gene transcription · molecular machines · nanotechnology · synthetic biology

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