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CAGE: Chromatin Analogous Gene Expression

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Abstract

Self-assembled nucleic acids perform biological, chemical, and mechanical work at the nanoscale. DNA-based molecular machines have been designed here to perform work by reacting with cancer-specific miRNA mimics and then regulating gene expression *in vitro* by tuning RNA polymerase activity. Because RNA production is topologically restrained, the machines demonstrate chromatin analogous gene expression (CAGE). With modular and tunable design features, CAGE has potential for molecular biology, synthetic biology, and personalized medicine applications.

Graphical Abstract

Keywords

CAGE; molecular machine; gene expression; RNA polymerase regulation

During gene expression, a base-4 DNA code is transcribed into a base-4 RNA code, which in turn is translated into a base-21 protein code. The primary and secondary gene products (i.e., RNA and proteins) are regulated by mechanisms that increase or decrease their production.^{1,2} DNA topology is a fundamental mechanism for controlling gene transcription.

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Notes

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^{3,4} For example, *Mycoplasma genitalium* transcription is highly regulated by changes in gene supercoiling.⁵ Other examples include regulating the accessibility of the promoter and/or operator regions in prokaryotes,^{5,6} as well as regulating histone and chromatin rearrangement in eukaryotes.⁴ To date, multiple experiments have shown gene regulation using synthetic small molecules,^{7,8} siRNA,^{9,10} viral vectors,^{11–13} bacterial vectors,¹⁴ and nanoparticles.^{15,16} While synthetic pathways that read and write nucleic acids by transcriptional and/or post-transcriptional factors have been reported,^{17–24} few are controlled by topological changes in the DNA structure.^{25,26}

Beyond biology, structural DNA nanotechnology^{27,28}—the rational design, synthesis, and characterization of complexes that are at thermodynamic equilibrium—exhibits elevated topological control using nucleic acids.²⁹ Topological control is exerted through molecular self-assembly of DNA origami³⁰ and/or bricks.³¹ As a molecular canvas, structural applications include organizing organic³² and inorganic³³ materials at the nanoscale for photonics,^{34–38} excitonics,^{39–41} and semiconductor fabrication.^{42–44} In comparison, dynamic DNA nanotechnology is the rational design, synthesis, and characterization of systems, that are far from equilibrium, using techniques such as toehold-mediated strand displacement,^{45–48} chemical reactions,^{49–51} and light-induced reactions.^{52,53} Principal applications include molecular computation,⁵⁴ chemical reaction networks,^{55–59} and molecular machines.^{60,61} When the structural and dynamic attributes of DNA are fully integrated, nanostructures can perform programmable state changes such as phase transformations and/or mechanical transformations. Emerging opportunities for integration include molecular biology,⁶² synthetic biology,⁶³ molecular computation,^{64–67} and personalized medicine,⁶⁸ which are enabled by DNA nanostructures' ability to sense,^{69,70} analyze,^{64,71–80} regulate,⁸¹ and transport^{82,83} nucleic acids and small molecules.

A promising application of dynamic DNA nanostructures, for synthetic biology, is the manipulation of biological processes. As three proofs of concept, encapsulation of therapeutic agents for drug delivery,^{84,85} antibody fragments to promote cell signaling,⁸⁶ and active enzymes to be delivered to HEK cells⁸⁷ have all been reported using DNA origami. In addition, when integrated into DNA origami, enzymatic activity and protease-dependent protein degradation have been enhanced and suppressed, respectively.⁸⁸ However, to date, dynamic DNA nanostructures have not been fully exploited for gene manipulation.

In this report, regulation of T7 RNA polymerase activity was achieved by modulating the availability⁸⁹ of the gene promoter regions in DNA nanostructures using toehold-mediated strand displacement.⁸⁰ Coined Chromatin Analogous Gene Expression, CAGE is similar to a Trojan horse because it both conceals and protects its payload from external forces prior to releasing it into the environment. Dually inspired by the accessibility and stability of information in chromatin, CAGE's function follows its structure. For example, its payload is deliberately designed to enable nonintegrating gene manipulation, which is vital when the risks associated with gene editing (i.e. domain swapping and shuffling) are significant.⁹⁰

As prototypes for this study, CAGE machines detected DNA mimics of specific miRNAs that signal for lung cancer. The DNA mimics were modeled after the lung adenocarcinoma miRNAs called hsa-miR-191 and hsa-miR-212.⁹¹ Once detected, the genetic payloads were

released from the CAGEs, which then initiated RNA transcription of specific DNA fragments called gene cassettes. The release of the payloads was monitored using Förster resonance energy transfer (FRET) between two dyes, one on each CAGE and the second attached to their respective payloads. Transcription of the gene cassettes was monitored via gel electrophoresis and qPCR. The detailed design, structural integrity, and information accessibility of the CAGEs are outlined below.

RESULTS

Three design iterations of the CAGE were engineered from DNA bricks or DNA origami, all of which are capable of integrating a gene cassette (Supporting Information S1,S2). While their mechanical structures are near identical, their molecular structures and payloads are unique. With minor changes to their staple libraries, each CAGE can accommodate an arbitrary gene cassette. One should note that regardless of the design, assembly process, and payload, the geometry and functionality of the machines are very similar.

The structure of each CAGE includes eight core DNA helices that self-assemble into a square lattice that conceals and protects the promoter region (Figure 1a,b). The promoter is located on the trapped strand (Figure 1b), which also includes (i) the gene cassette that is external to the CAGE, (ii) a universal linker that couples the gene cassette to the trapped strand using a *Bgl*II restriction site, and (iii) two sequence-specific detection sites, for the miRNA mimics, that release the trapped strand from the CAGE machine. The restriction site is where genetic content is grafted into the trapped strand during pre- or post-CAGE synthesis (Supporting Information S2). In addition, the miRNA detection sites are modular, and to change them, the anchor strands in Figure 1c,d are replaced. The detection regions have toeholds with seven bases that drive strand displacement reactions forward between the miRNA mimics and their respective trapped strand (Figure 1d and Supporting Information S3), releasing the trapped strand from the CAGE. Although multiple CAGEs were designed, synthesized, and tested in this report, a representative data set is presented here. For the additional CAGE data sets, please see the Supporting Information.

For FRET, Cy5 and Cy3 dyes were attached to one CAGE core helix and upstream of the promoter region on the trapped strand, respectively (Figure 1b). When the trapped strand is integrated into the CAGE (inactive state), the dyes are proximal and cause efficient FRET. When the trapped strand is released from the CAGE (activated state), the distance between the dyes increases, the FRET efficiency decreases, and the promoter region becomes accessible to the RNA polymerase (Figure 1a,c and Supporting Information S4, S5). CAGE activation is achieved by using the miRNA mimics or their corresponding RNA strands (Supporting Information S4,S5). In contrast, replacing the mimics with noncomplementary DNA or RNA dummy strands did not greatly affect the FRET signal strength nor significantly release the trapped strand, rendering the CAGE inactive (Supporting Information S4).

For transcriptional regulation, T7 RNA polymerase was added into the buffer solution with the activated and inactive CAGE machines and a time-dependent study was performed. After deactivation of RNA polymerase, DNaseI digested the DNA, and acrylamide gel

electrophoresis was performed to characterize the RNA production. Availability of the promoter region, when the trapped strand is released from the CAGE machine, leads to transcription of the gene cassette, and hence production of the target RNA. In contrast, inaccessibility of the promoter region, when the trapped strand is integrated into the CAGE machine, leads to suppression of RNA production. Because efficient RNA transcription from the activated machine caused turbidity of the reaction medium, differences in total RNA production between the inactive and activated CAGE were visible by eye and when stained with SYBR Safe dye (Figure 2a). Gel electrophoresis confirmed that the transcription efficiency of the activated CAGE is greater than the inactive CAGE (Figure 2b). When statistically compared, the band intensities of the activated samples were also greater than the inactive samples (Figure 2c and Supporting Information S6).

CAGE activation is a function of the miRNA mimics concentration and hence is tunable. For example, as the mimic concentration decreases so does RNA transcription (Figure 2d, Supporting Information S7). Eliminating the mimic toeholds sequentially suppressed strand displacement, CAGE activation, and RNA production (Figure 2e). Modularity was shown through successful operation of the independent CAGEs (Supporting Information S6), each possessing a unique gene cassette.

For transcribed RNA quantification, probe-based qPCR was performed and the rate of each reaction was calculated. In addition, probe-based qPCR was used to evaluate the specificity of RNA transcription and demonstrated that the correct target transcripts were produced. Relative activity of the RNA polymerase for each experimental data set was calculated in comparison to the enzyme activity in the control sample. Throughout the time-course study, relative RNA polymerase activities of the activated CAGEs were significantly greater than the inactive machines (Figure 3 and Supporting Information S8). The rate of RNA transcription for activated and inactive CAGEs were calculated to be $2.19 \times 10^{-16} \text{ M s}^{-1}$ and $3.53 \times 10^{-19} \text{ M s}^{-1}$, respectively. While transcription of the gene cassette for the inactive CAGE was significantly lower, the leakage reaction was nontrivial. Hypothesized sources of leakage are released trapped strands from malformed and/or damaged CAGEs during synthesis, purification, and/or pipetting.

DISCUSSION

Gene regulatory systems are influencing molecular biology, synthetic biology, and personalized medicine.⁹² Presented here, CAGE was modeled as a molecular machine for *in vitro* applications. Akin to a Trojan horse, CAGEs conceal and protect their payload from external forces prior to releasing them into the medium. Inspired by the accessibility and stability of information in chromatin, CAGEs are potentially safe alternatives to viral and bacterial gene expression systems. Because of its conditional attributes, off-target interactions are limited and undesirable gene expression is reduced. Unlike conventional viral and bacterial systems, CAGE nanomachines are capable of nucleic acid detection and gene regulation. As a proof of concept, conditional transcription of arbitrary genes was achieved in response to interacting with lung adenocarcinoma miRNA mimics. The RNA transcription rate was reduced ~1000 times between the activated and inactive CAGEs. Future research is suggested to (i) study the *in vivo* performance of the CAGE, and (ii)

conjugate cell-receptor ligands to the CAGE as a specific and targeted gene regulation platform.

METHODS

Design Principles and Construction of CAGES

The CAGE machines were designed similarly to previously described reports.^{46,80} The structural details of the nanostructures were drawn either using caDNAno⁹³ or NanoEngineer. The final geometries of designed structures were analyzed using CanDo⁹⁴ and Gromacs.⁹⁵ Detailed description of designing procedure can be found in Supporting Information S1. Sequences of the oligos were designed using NUPACK⁹⁶ (Supporting Information S9). Oligos were ordered from Integrated DNA Technologies (IDT), where the chemically modified DNA strands were ordered as HPLC purified. List of all DNA strands, their sequences, and their roles are described in Supporting Information Appendix.

Construction, Configuration Change and FRET Studies of the CAGES

The CAGE machines were made by mixing oligos in a 1× TAE buffer (1× TAE; pH, 8.0; 40 mM MgCl₂), heating the mixture to 90 °C for 1 min and reducing the temperature to 20 °C over a course of 60 h. Purification of the samples were performed using Amicon Ultra-0.5 100 kDa Centrifugal Filter [EMD Millipore] (Supporting Information S2). To produce activated or inactive CAGE machines, 63 μL of ~500 nM CAGE machine was added to Eppendorf tubes containing 2 μL of ~100 μM of miRNA mimics or dummy DNA oligos, respectively (Supporting Information S3). Mixtures were gently pipetted up and down 15 times. FRET analyses were performed as described elsewhere⁸⁰ and in Supporting Information S4 using a Fluoromax 3 fluorometer [Horiba Jobin-Yvon]. Signal intensities at 494, 565, and 665 nm were recorded as references to normalize the amount of materials for upstream reactions (Supporting Information S8).

In vitro RNA Transcription, Gel Electrophoresis, and qPCR

The T7 RNA polymerase was selected for the RNA polymerase activity studies because it is commercially available, promoter specific, transcribes downstream of the promoter region, and requires a double-stranded DNA promoter and Mg²⁺ as its cofactor.⁹⁷ It also has high fidelity with an error rate of 1 in 0.5 × 10⁴ bases.⁹⁸ Because of its size—6.1 nm in diameter⁹⁹ and 98.8 kDa in molecular weight¹⁰⁰—our assumption was that the enzyme is larger than the ~3 nm CAGE pores and hence would not penetrate the structure to access the promoter. RNA transcription was performed using TranscriptAid T7 High Yield Transcription Kit [Thermo Fisher Scientific] or OPTIZYME T7 RNA Polymerase [BioReagents] in a total of 100 μL volume containing 10 μL (~4.8 pmol) of CAGE machines, and 2 μL of RiboLock RNase Inhibitor [Thermo Fisher Scientific]. Control samples consisted of the unassembled trapped strand that contained the gene cassette and the double-stranded promoter region. RNA polymerase activity was triggered by either incubating the 15 μL aliquots of mixtures at 37 °C for 5, 15, 30, 60, 90, and 120 min or taking 15 μL of the 100 μL mixtures that were incubated at 37 °C at the aforementioned time intervals. For the control sample RNA transcription was conducted during a 120 min reaction time. The aliquots were spun down briefly and transferred to –20 °C, immediately.

To deactivate T7 RNA polymerase, after all the aliquots were ready, the tubes were heated at 80 °C for 10 min and rapidly cooled down to 4 °C, following by the addition of 1 μ L of DNaseI and 1 μ L of RiboLock RNase Inhibitor to each tube, incubated at 37 °C for 30 min, and incubated at 75 °C for 15 min to inactivate DNase I. Gel electrophoresis was performed using 20% acrylamide gel (Supporting Information S6). For qPCR studies, all samples were diluted by a factor of 2 and the reverse transcription reaction was carried out for 10 μ L of samples using Maxima Reverse Transcriptase [Thermo Fisher Scientific]. The cDNA samples were further diluted by a factor of 1000, and probe-based qPCR reactions were performed using Luminaris Color Probe qPCR Master Mix [Thermo Fisher Scientific] in a total volume of 15 μ L in LightCycler 96 System [Roche Life Science] (Supporting Information S8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) General CAGE illustration including its overall structure and operation. (b) Trapped strand contains gene cassette that is external to the CAGE. Other segments of the trapped strand include a universal linker that connects the gene cassette to the trapped strand using a restriction site for enzyme *BglII*, two sequence-specific detection sites for the miRNA mimics that release the trapped strand from the CAGE, T7 RNA polymerase promoter, and a Cy3 dye. (c) Cross section when the trapped strand is integrated into (top) and released from (bottom) the CAGE. FRET is maximized and minimized when the trapped strand is integrated into and released from the structure, respectively. (d) The miRNA mimics initiate toehold-mediated strand displacement of the trapped strand at domains a' and f', which releases the anchor strands. The mimics were modeled after the lung adenocarcinoma

specific miRNAs called hsa-miR-191 and hsa-miR-212.⁹¹ For scale in part d, each dash line in regions 1 and 2 represents four bases.

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Figure 2.

Representative analysis of the CAGE machine performance in RNA transcription. (a) Left image: the turbid solution (tube 2; activated CAGE machine) contains greater amounts of RNA. Right image: the samples were stained with SYBR Safe. Tube 1 represents RNA transcribed from the CAGE in its inactive state (in the presence of the DNA dummy strands). Tube 2 represents RNA transcribed from the CAGE in its activated state (when the miRNA mimics were present). (b) Gel electrophoresis image of produced RNA in a time-course study. Control sample contained the RNA produced in a 120 min reaction from unassembled trapped strand. (c) Calculated relative signals of the gel electrophoresis bands in a time-course study. Relative signals were calculated as relative signal = (signal intensity at given band)/(signal intensity for the band of control sample (trapped strand)). (d) RNA transcription regulation was tuned as a function of the miRNA mimics concentration. The relative concentration of the miRNA mimics were chosen in comparison to the concentration of the CAGE machine. (e) Efficient activation of RNA production is depended on toehold-mediated trapped strand release. The short miRNA mimics lacked the complementary sequences of the miRNA detection site toehold regions and did not effectively activate the RNA transcription reaction. RNAP relative activities of each reaction in panels d and e were calculated in comparison to the highest amount of RNA produced in each experiment (dark green bars).

Figure 3. qPCR analysis of RNA transcription for inactive and activated CAGE machines in a time-course study. The produced RNA was reverse transcribed to cDNA, and qPCR was performed to quantify the amount of transcribed RNA. The once trapped strand is disassembled from CAGE, transcription of the target gene cassette led to a few orders of magnitude increase in the RNA production. Relative activities were calculated as RNP relative activity = amount of RNA produced at a given time/amount of RNA produced for control sample (trapped strand) over 120 min.