A modular DNA signal translator for the controlled release of a protein by an aptamer

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ABSTRACT

Owing to the intimate linkage of sequence and structure in nucleic acids, DNA is an extremely attractive molecule for the development of molecular devices, in particular when a combination of information processing and chemomechanical tasks is desired. Many of the previously demonstrated devices are driven by hybridization between DNA ‘effector’ strands and specific recognition sequences on the device. For applications it is of great interest to link several of such molecular devices together within artificial reaction cascades. Often it will not be possible to choose DNA sequences freely, e.g. when functional nucleic acids such as aptamers are used. In such cases translation of an arbitrary ‘input’ sequence into a desired effector sequence may be required. Here we demonstrate a molecular ‘translator’ for information encoded in DNA and show how it can be used to control the release of a protein by an aptamer using an arbitrarily chosen DNA input strand. The function of the translator is based on branch migration and the action of the endonuclease FokI. The modular design of the translator facilitates the adaptation of the device to various input or output sequences.

INTRODUCTION

In the past few years, the information encoding and strand recognition capabilities of DNA have been utilized for the realization of a variety of nanoscale DNA-based devices. DNA conformational changes have been shown to result in rotatory (1,2), stretching (3–12), and even translatory (13–18) movements. DNA devices have also been combined with functional nucleic acids such as ribozymes (9,17,19) and aptamers (20,21). Apart from chemomechanical action, DNA has also been demonstrated to be capable of a variety of information processing tasks (22–28). One of the more recent developments here is the realization of autonomous molecular automata performing, e.g. simple logical computations (29–36).

To implement more complex functions into molecular systems, it is of great interest to combine the computational and chemomechanical capabilities of these devices by linking them together into artificial reaction networks. As one example, we demonstrated recently how the operation of a DNA nanomechanical device can be controlled by mRNA signals transcribed from regulatory ‘genes’ (37,38). However, in this case both the ‘gene’ and the DNA device were artificially constructed and the sequences could be chosen freely. In a more realistic application one would like to trigger the action of a DNA-based device with an arbitrary DNA or RNA input signal. As an example, consider the release of a specific molecule bound to a DNA aptamer in response to the presence of a particular mRNA molecule. In general, the sequence of the mRNA (indicating the expression of a gene) will be completely unrelated to the DNA aptamer sequence. There are several possible solutions to this problem. One solution commonly found in nature is ‘allosteric regulation’. Here two spatially separated binding sites on an enzyme communicate with each other via a conformational change triggered by the binding of an effector to one of the binding sites. Such a principle has been utilized previously for the construction of allosteric ribozymes or aptazymes [e.g. (39–41)], and also recently for the DNA-controlled release of a small molecule by an aptamer (21). In some cases, however, allosteric regulation may not be an option—e.g. when the binding capacity of an aptamer or the activity of a ribozyme is affected too strongly by sequence modifications and the introduction of a regulatory part is not feasible. For ribozyme regulation, one approach is the ‘expansive regulation’ strategy developed by Wang et al. (42,43). Here the activity of a ribozyme is regulated by an effector molecule stabilizing the ribozyme–substrate complex. To regulate protein release by an aptamer, in the present work an alternative approach is taken in which a DNA or RNA input signal is translated into the desired output signal by a molecular translation device. One possible realization of such a translator is the molecular automaton recently

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introduced by Benenson et al. (31) which is based on the action of the restriction endonuclease FokI. As a type II-S endonuclease, FokI cuts 9 and 13 nt away from its 5 nt recognition sequence, and therefore the 4 nt sticky end created by this process is unrelated to the recognition sequence itself. In Ref. (31), the recognition sequence for FokI was hidden within an internal loop of a DNA double strand. In the presence of input strands (‘disease indicators’), the FokI recognition site was completed and a DNA ‘drug’—the product of cleavage by FokI—was administered. We here use the FokI system as a DNA signal translator to trigger the release of a protein by an aptamer device using an arbitrarily chosen DNA input signal. In contrast to Ref. (29), the DNA signal translator used here consists of two hairpin structures which contain the FokI recognition site and the effector sequence, respectively. The FokI site is inactivated by initially hybridizing the hairpin to a ‘protection’ strand. The translator is activated through the removal of the protection strand by a DNA input strand with a sequence completely unrelated to the effector or aptamer sequence.

MATERIALS AND METHODS

Materials

FokI (4 U/µl) was obtained from New England Biolabs, human α-thrombin in solution (200 µM) from Cell Systems, Germany. SYBR Gold nucleic acid stain was from Molecular Probes (Invitrogen), all other chemicals mentioned from Sigma–Aldrich. DNA strands were synthesized by biomers. net, Germany. The sequences of the oligonucleotides for the construction of the device (IN, CO, PR, OUT, APT) are given in Table 1. For fluorescence measurements a doubly labeled aptamer specific for the protein thrombin and extended by a 12 nt toehold was used (APT), similar to the aptamer device reported before (20,44). DNA strands were designed to minimize cross-hybridization and to have favorable ratios of melting temperatures in the different states of the device. The sequence generator software DNasequenceCompiler (45) was used for creating the sequences according to these specifications, the nucleic acids folding program RNAstructure (46) for checking the secondary structures.

Device preparation and gel electrophoresis

For gel electrophoresis experiments, DNA strands were diluted to 1 µM in the hybridization buffer (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 at 25°C). The concentration of the strands was determined from their OD 260.

A typical reaction mixture contained 0.5 µl CO, 2.5 µl of PR, 4 µl of OUT and 4 U/10 µl FokI. The addition of 3 µl of IN started the reaction. All samples were run on native 18% polyacrylamide gels in TBE (Tris–borate–EDTA, pH 8.3) buffer at 100 V/cm for 1.5–2 h and stained with SYBR Gold using the manufacturer’s protocol and imaged under UV illumination.

Fluorescence measurements

Fluorescence resonance energy transfer (FRET) experiments were performed with the doubly labeled DNA aptamer strand APT. In its folded G quadruplex state (compare Figure 5) its two ends are in close proximity and energy transfer from fluorescein to TAMRA occurs efficiently. When APT is stretched due to hybridization with the output strand, FRET between the fluorophores is less efficient and fluorescein fluorescence is increased. The fluorescence signal therefore directly monitors the progress of the hybridization reaction of the output strand with the target strand APT. For the fluorescence experiments, all DNA strands were initially diluted to 10 µM. For the enzyme reaction, buffer composition, ratio of the strands, time and temperature was the same as for the gel assays. The FokI reaction was performed in a volume of 50 µl containing 20 U of FokI and terminated before the FRET measurements.

For the measurements, the aptamer strands were diluted in 1 ml aptamer buffer (20 mM Tris–HCl, 150 mM NaCl, 10 mM MgCl₂ and 10 mM KCl, pH 8.0) to a final concentration of 75 nM.

FRET measurements were performed on a spectrofluorometer (Fluorolog-3, Jobin-Yvon, Munich, Germany). Fluorescence was excited at 490 nm and recorded at 515 nm, with excitation and emission bandwidths of 10 nm. The temperature was kept constant at 23°C.

Thrombin–aptamer complexes

For experiments with thrombin, the aptamer strand was mixed with thrombin in a ratio of 1:2. Aptamer and thrombin were incubated for 30 min at 23°C in a reaction volume of 50 µl before FRET measurements were conducted in a sample volume of 1 ml.

RESULTS AND DISCUSSION

Operation principle of the DNA transducer

The operation principle of the transducer is schematically depicted in Figure 1. It is based on three processes which have been utilized previously for the operation of other DNA nanodevices and automata: (i) DNA branch migration (3); (ii) the specific DNA cleaving properties of the restriction endonuclease FokI (29); and (iii) the inhibition of hybridization between complementary strands by secondary structure formation (47). As shown in Figure 1A, the DNA output signal

| Strand | Sequence (5’–3’) |
|--------|-----------------|
| IN     | GATTGCGAAAAAGAAGTATGAGATAATGTACAC |
| CO     | GCTGCACTCAGATTGCGGAAAAGAAGTATGAGATCGG ATG |
| PR     | GTGACATTATCTCATACCTTCTCTTCCGCGAATCGGA |
| OUT-1*| CAAAGCGAATTGTGCAATCCACCAACGCGGAGGTAAATGCC |
| ACTT   | ACTT |
| OUT-1*| CGCATTCATCCACCAACGCGGAGGTAAATGCC |
| OUT-2*| CAAAGCGAATTGTGCAATCCACCAACGCGGAGGTAAATGCC |
| GTCGAC |
| OUT-2*| CGACTAACCACCCACACGCGGAGATGAACCTA |
| OUT-3*| CCCACCAACGCGGAGATTTGTCGCTAA |
| OUT-3*| CGACCAACGCGGAGATTTGTCGCTAA |
| APT    | TAAGGTTCAATCTCGGATTGTGATGTTGATGTTG |

Table 1. Sequences of the DNA strands used
initially is in an inactive state—it is forced into a small hairpin loop in which hybridization with its complement is inhibited (31,47). The stem of the hairpin has a sticky end which is complementary to the sticky end of a ‘connector strand’ (CO) which can also assume a hairpin conformation. However, initially the connector strand is forced into a duplex structure by a ‘protection strand’ (PR) in which it cannot hybridize to the inactive output strand. The protection strand in turn is equipped with a single-stranded ‘toehold’ (3) at which the input DNA strand (IN)—which is to be translated into an active output strand—can attach. The input strand can displace the protection strand from the connector strand by branch migration, producing a double-stranded waste product (IN-PR, Figure 1B). The released connector strand can now fold into its hairpin structure and hybridize to the inactive output signal. The sequence of the connector strand CO is chosen in such a way that in the folded state it contains the FokI recognition sequence (5’-GGATG-3’/3’-CCTAC-5’). FokI binds to the complex formed by connector and inactive output and cleaves the output strand 9 and 13 bases downstream from the FokI recognition site. As has been shown previously by Benenson et al. (30), FokI can cleave such a construct even without ligation of the two substrate duplexes. The result of the cleavage reaction is shown in Figure 1C.

The connector strand leaves the reaction unaltered, whereas the stem of the output signal strand has been shortened (OUT becomes OUT*). The loop conformation of the output strand is therefore less stable and breaks open which makes the output DNA sequence available for hybridization with a downstream target sequence. In the present example, the activated output signal can bind to an aptamer-based DNA device and trigger the release of the protein thrombin. The release of the protein is achieved by the competition for binding between the output strand and the protein to the aptamer. The output strand sequence therefore necessarily has to be chosen (at least partly) complementary to the aptamer sequence. Using the DNA translator, an arbitrarily chosen DNA input strand can be translated into this specific output strand.

**Design of the input section**

The various sections of the transduction device are indicated in Figure 2. In order to emphasize the modularity and generality of our approach, we here describe the design of the input and output sections in terms of an ‘algorithm’. (i) Given is the input signal strand IN which is to be transduced into a different sequence. This input signal can be of any sequence with length of >20 nt, it does not have to be extended...
or altered. (ii) The protection strand PR is the complement of strand IN and has to be augmented at the 3’ end with the three bases ‘GGA’ contained in the FokI recognition sequence. This prevents the binding of FokI to dimers which may be formed by two protected connector strands in the inactive state of the device. (iii) The connector hairpin loop CO can be derived from IN in the following way: cut off 8–10 bases from the 3’ end of IN, as they are used as toehold sequence for the displacement of PR from CO. (iv) Add the reverse complement of the first three bases of the 5’ end to the 3’ end, this acts as a spacer between the FokI binding site and the loop (compare section B of Figure 2). We experimentally verified that binding of FokI is obstructed if <3 bp are located upstream between the recognition sequence and the first bases of the loop. (v) Next add the FokI recognition sequences to the ends: CATCC to the 5’ end and GGATG to the 3’ end (C in Figure 2). (vi) As a last step, a sticky end has to be added to the connector hairpin (D in Figure 2). Since FokI cleaves asymmetrically with an overhang of 4 bases at the 5’ end, it is best to attach the sticky end at the 5’ end as well. The lower limit of the length of the sticky is set by the temperature at which the intermediate dumbbell structure (Figures 1B and 2) has to be stable. The upper limit is given by the distance between the recognition and the restriction site of FokI on the 5’ end, i.e. 13 bases. Reasonable values are between 4 and 8 bases (compare next paragraph).

Design of the output section
The complement of the sequence of the target strand (e.g. an aptamer-based device) has to be incorporated into the stem and/or the loop of the output section (E and F in Figure 2). In the inactive state (before cleavage by FokI), hybridization between the output loop and the target strand should be kinetically inhibited, whereas in the active state this reaction should be fast. The stem of the output loop (E in Figure 2) therefore has to be sufficiently long to be stable in its uncleaved form and short enough to break open after restriction by FokI. Furthermore, the opening of the loop (F in Figure 2) has to be kept small enough to prevent the complementary strand from threading through (47). Several design variations based on these considerations are described below. The design of the sticky end connecting the input and output section of the device (D in Figure 2) has to ensure efficient cleavage by FokI. As described in Ref. (30), cleavage without prior ligation is possible for short sticky ends with a high CG-content. In experiments at various temperatures we found that the restriction reaction was successful with a 5 nt sticky end for temperatures up to 20°C. It has been found previously (30) that

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**Figure 2.** Core of the device composed of strands CO and OUT from Figure 1. Section A contains the part that is complementary to the protection strand PR. C is the FokI recognition sequence, spacer section B is needed to ensure that the binding of the enzyme is not disturbed by the loop A. Section D connects the two loops, it should be short and rich in GC pairs to improve FokI performance across the nicks. Line E indicates the FokI cleavage site, with the output F as result of the translation process.

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**Figure 3.** Operation of the device characterized by gel electrophoresis. Lane 1 contains output (OUT) and connector (CO) strand in the absence of FokI. Lane 2 contains OUT and CO with FokI added, leading to the appearance of the cleavage products OUT*, OUT#. The latter is due to incomplete cleavage of the output strand (see text). Lane 3 contains protected connector strand (CO-PR) and OUT in the absence of FokI; lane 4 contains the same strands plus FokI added. Almost no cleavage reaction occurs in this situation. In lane 5, the input strand IN was added to the mixture contained in lane 4, leading to cleavage of the output strand. A new band appears for the ‘waste’ product IN-PR. Lane 6 contains the pre-cleaved output strand OUT* for comparison.

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**Experimental proof of the mechanism**
The basic operation of the DNA translator was characterized in gel electrophoresis experiments. The gel image displayed in Figure 3 clearly demonstrates that FokI can cleave the output loop OUT in the presence of the connector loop CO even without ligation. It also shows that cleavage is inhibited when the connector strand CO is bound to the protection strand PR. When a removal strand IN is added to the reaction mixture, the protection strand is displaced from the input loop by branch migration and the restriction reaction starts. This can be monitored by the appearance of a band for the ‘waste’ duplex (IN-PR) and for the restricted output strand OUT*. The intensity of these bands can also be used to obtain the transfer function of the device which relates the concentration of the output strand to that of the input strand (Figure 4). For the strand concentrations used in this particular experiment (compare Materials and Methods), the I/O relation is slightly sigmoidal (Figure 4B). The linear relationship between band intensity and DNA concentration in the relevant concentration range used was verified in a separate experiment.

**Influence of design parameters**
As described above, several design parameters are critical for the operation of the DNA transducer: On the input side, the length of the spacer sequence (section B in Figure 2) is important. When shorter than 2 bp, the distance of the FokI recognition site and the loop is too small to accommodate the FokI enzyme. When larger than 4 bp, the formation of dimers between protected input strands leads to false positive reactions. The ideal length therefore turns out to be 3 bp. To reduce side reactions due to dimer formation, it is necessary that the protection strand not only overlaps with one side of the spacer sequence (B in Figure 2), but also with part of the recognition site (C in Figure 2). The length of the linker sequence

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The intensity of the product band as a function of input strand concentration.

6

loop sizes, whereas OUT-1 and OUT-3 have different stem

free-running DNA nanomachines.

context of molecular beacons and also as a concept for fuel-

ment. Similar effects have been studied extensively in the

sequence more accessible for hybridization with its comple-

ment should occur efficiently. Experimentally, three different
designs for the output section result in completely different

outcomes. As can be judged from Figure 5, the three different
strand designs differ in stem length and loop sizes (triangles, OUT-1,
14 bp stem/10 nt loop; OUT-1*, 6 bp stem/10 nt loop; OUT-2, 11 bp (3 bp) stem/20 nt
loop; OUT-3, 11 bp (3 bp) stem, 7 nt loop, (for sequences see Table 1)]. To study the hybridization kinetics, we first per-
formed experiments with synthesized output strands with
appropriately shortened sequences to mimic the behavior of
the FokI reaction products. The hybridization kinetics of the
three different output loops in their cleaved and uncleaved
form was determined in FRET measurements. In all cases the
target sequence for hybridization was the doubly labeled
aptamer strand APT. Hybridization between the output strand
and APT results in a strong increase in fluorescence, as the
FRET donor (fluorescein) and acceptor (TAMRA) are spa-
tially more separated from each other in the duplex form
than in the folded single-stranded G quadruplex form of
APT. As can be judged from Figure 5, the three different
designs for the output section result in completely different
hybridization kinetics. This is due mainly to two effects. First,
hybridization of a DNA strand with a stable DNA hairpin loop
is extremely slow. To form a double helix, the DNA strand has
to form a few initial base pairs with bases in the loop and
then wind through the loop hole. Depending on the size of the
loop, this can be very slow or even impossible on an experi-
mental time scale. It also has to be considered that hybridiza-
tion of a DNA strand to a hairpin loop structure is reduced even
further when no end of the strand is able to bind within the loop
and break open the stem through branch migration (47).

The second important parameter—the stem length—has
influence on the stability of the loop conformation. A short
stem will break open occasionally and make the DNA loop
sequence more accessible for hybridization with its comple-
ment. Similar effects have been studied extensively in the
context of molecular beacons and also as a concept for fuell-
ing free-running DNA nanomachines.

OUT-2 and OUT-3 have the same stem lengths and different
loop sizes, whereas OUT-1 and OUT-3 have different stem

lengths and comparable loop sizes. As can be seen in Figure 5,
due to the 20 nt long loop region, OUT-2 already strongly
hybridizes with the target sequence in the ‘inactive’ state
before cleavage by FokI. In contrast OUT-1 and OUT-
3—with the small loops—only show a low level of unwanted
hybridization with the target. After cleavage, all output strands
hybridize well with the target sequence. Here, OUT-1*
displays slower hybridization kinetics than OUT-2* and
OUT-3* as it still has a considerable stem length (6 bp)
before cleavage.

Performance of the device

In Figure 6, the hybridization kinetics of the full translation
device containing FokI, PR, CO, OUT-3 with the target strand
APT is shown. Before the addition of the input signal IN, the
spurious hybridization reactions are at roughly the same level
as in a ‘clean’ system containing only OUT-3. After addition
of the input signal strand IN, the degree of hybridization to the
target is enhanced several fold, showing the successful per-
formance of the DNA translation device. The efficiency of
hybridization is comparable with that with the pre-cleaved
strands OUT-3*. Also shown is a test experiment, in which
only the arbitrarily chosen strand IN is added to the aptamer
device APT. This input strand in fact does not display any
interaction with the target at all. This demonstrates that the
device in fact works as a signal translator. By the action of the
translator an otherwise non-reactive input strand can influence
the hybridization with a target sequence.
Translator-controlled release of thrombin by the aptamer device

As an application of the signal translator concept, we demonstrate how the release of a protein by an aptamer-based DNA device can be controlled by an arbitrarily chosen DNA input signal. In Figure 7, the hybridization of the DNA translator reaction mixture with the DNA aptamer device APT bound to the protein thrombin is shown with and without the addition of the input strand IN. The initial fluorescence level is decreased to about 80% in comparison with Figure 6 (I_{0}^{bound} = 0.78 I_{0}), as the folded G quadruplex structure of APT is now bound to the protein, leading to a stronger FRET effect—i.e. APT acts as a sensor for thrombin (48,49). Upon hybridization of the output strands with the aptamer device the fluorescence intensity increases. In a simplified two-state model, the total fluorescence signal is given as

\[ I(t) = \xi(t) I_{\text{released}} + [1 - \xi(t)] I_{\text{bound}}, \]

where \( \xi(t) \) is the fraction of aptamers which have released their protein. \( I_{\text{released}} \) is the maximum fluorescence value obtained for aptamer beacons completely hybridized to the output strands and can be estimated from the limiting value \( I_{\text{released}} = 5.8 I_{0} \) (from Figure 6), where \( I_{0} \) is the initial fluorescence value for APT in the absence of protein. In Figure 7, the degree of protein release \( \xi(t) = [I(t) - I_{\text{bound}}]/[I_{\text{released}} - I_{\text{bound}}] \) is plotted. In the presence of the protein, this process is much slower than the hybridization of output strands with the aptamer without thrombin. The side reaction with the ‘inactive’ output strand OUT-3 already leads to a slight protein release. However, as desired the action of the DNA translator in response to the input strand IN leads to a much more efficient protein release. In principle, the side reaction could be completely suppressed if two distinct input signal strands were used and a correspondingly longer stem region for the inactive output strand (31).

CONCLUSIONS

We have demonstrated how a molecular signal translation device based on DNA hairpin loops and the action of the restriction endonuclease FokI can be used to release a specific DNA output strand in response to an arbitrarily chosen input strand. This translator can be used to uncouple nucleic acids-based signal and effector molecules and therefore link together molecular processes which produce or require specific DNA or RNA strands. Such situations may arise in DNA-based self-assembly, when the temporal or logical order of several self-assembly steps is to be controlled or when reaction networks of DNA-based nanodevices are to be constructed. Similar situations are of considerable interest in the context of intelligent biosensing or even drug delivery, when a specific compound is to be released in response to the presence of a certain nucleic acids molecule, e.g. mRNA. As a specific example, we demonstrated how the release of a protein by an aptamer-based nanodevice can be controlled by an arbitrarily chosen DNA strand which is translated by the molecular translator into a protein-releasing effector sequence.

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REFERENCES

1. Mao,C.D., Sun,W.Q., Shen,Z.Y. and Seeman,N.C. (1999) A nanomechanical device based on the B–Z transition of DNA. Nature, 397, 144–146.

2. Yan,H., Zhang,X.P., Shen,Z.Y. and Seeman,N.C. (2002) A robust DNA mechanical device controlled by hybridization topology. Nature, 415, 62–65.

3. Yurke,B., Turberfield,A.J., Mills,A.P., Simmel,F.C. and Neumann,J.L. (2000) A DNA-fuelled molecular machine made of DNA. Nature, 406, 605–608.

4. Simmel,F.C. and Yurke,B. (2002) A DNA-based molecular device switchable between three distinct mechanical states. Appl. Phys. Lett., 80, 883–885.

5. Li,J.W., Zhang,X.P., Shen,Z.Y. and Seeman,N.C. (1999) A nanomechanical device based on the B–Z transition of DNA. Nature, 397, 144–146.

6. Alberti,P. and Mergny,J.L. (2003) DNA duplex-quadruplex exchange as the basis for a nanomolecular machine. Proc. Natl Acad. Sci. USA, 100, 1569–1573.

7. Liu,D.S. and Balasubramanian,S. (2003) A proton-fuelled DNA nanomachine. Angew. Chem. Int. Ed. Engl., 42, 5734–5736.
27. Su, X.P. and Smith, L.M. (2004) Demonstration of a universal surface

28. Schmidt, K.A., Henkel, C.V., Rozenberg, G. and Spaink, H.P. (2004) DNA computing using single-molecule hybridization detection. Nucleic Acids Res., 32, 4962–4968.