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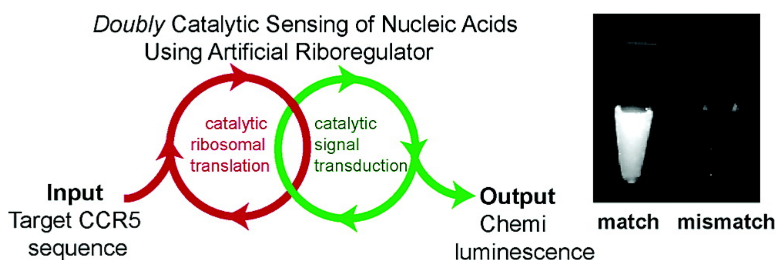

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Doubly Catalytic Sensing of HIV-1-Related CCR5 Sequence in Prokaryotic Cell-Free Translation System using Riboregulator-Controlled Luciferase Activity

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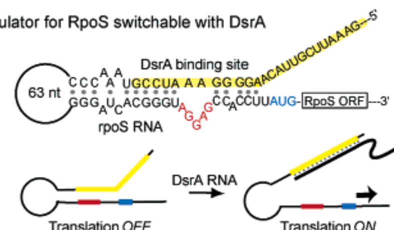
As the number of identified genetic markers continues to grow, there has been much current interest in rapid and simple gene sensing.[1] One of most successful and general methods of homogeneous nucleic acid sensing so far reported[2] is the use of molecular beacons (MBs),[3] which are hairpin-shaped oligonucleotide probes with an essential target-complementary sequence held in a loop domain linked to a FRET pair at the 5′- and 3′-ends or, in newer versions, to a ribozyme/DNAzyme sequence[4−8] or a redox center.[9] The recent inhibitor−DNA−enzyme conjugate[10] may also be mechanistically related to MBs. Binding of the target results in opening of the hairpin structure to restore fluorescence or to switch on the enzymatic (ribozyme/DNAzyme[4−8] or proteinous[10]) or electrochemical[9] *catalysis*, thus allowing signal amplification.[11] The present work is concerned with linkage of the MB motif to a protein translation regulator for multiply catalytic gene sensing. We report here that an HIV-related chemokine receptor sequence can be sensed by chemiluminescence using an unmodified RNA or DNA probe with a sensitivity of ≤50 fmol and a single nucleotide selectivity.

Regulation of translation with a small RNA is a naturally occurring function.[12] The RpoS mRNA contains a hairpin-shaped structure to sequester the ribosome binding site (RBS) (shown in red in Figure 1a).[13] Binding of a small RNA DsrA at the anti-RBS region (in yellow) leads to opening of the hairpin structure, makes the RBS domain accessible, and hence triggers translation of the RpoS protein (Figure 1a).[13] Recently, Collins et al. have demonstrated that translation of a simplified mRNA (riboregulator) cis- or hairpin-repressed in this manner can be activated by an external, that is, transacting small RNA complementary to the stem-loop region including the anti-RBS.[14] Inspired by these findings, we designed an MB-type riboregulator capable of multiple amplification. Luciferase was a sensing output of choice because of the high sensitivity and good linearity of the chemiluminescence assay based thereupon (Figure S1). Thus, the riboregulator (mRNA) probe[15] (Figure 1b) contains the reporter gene for luciferase and, upstream thereof, a regulatory hairpin domain composed of an 8 bp stem (bp = base pair) involving a 6 nt RBS (in red) (nt = nucleotide) and a 19 nt loop, 16 nt of which (yellow) is complementary to the 620−635 region (yellow) of the human CC chemokine receptor 5 (CCR5). Binding of 25-mer CCR5 oligodeoxynucleotide (ODN^{full}) as the target with concomitant liberation of RBS would ignite ribosomal catalytic production (translation) of luciferase which, in turn, is capable of catalytic production of chemiluminescence. Accidentally, the underlined 5 nucleotides in the 3′-end of the target are complementary, if the central A is bulged, to the anti-RBS region (UCCU). The former may thus serve as an anti-(anti-RBS) to cooperate with the designed 16 bp target−probe hybridization to open the hairpin in competition with the intrastrand 8 bp or (8 + 5) bp[16] (Figure S2) stem formation.



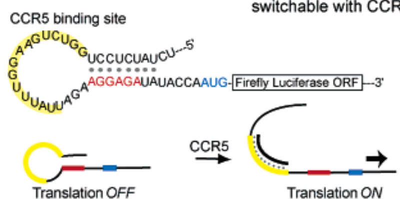
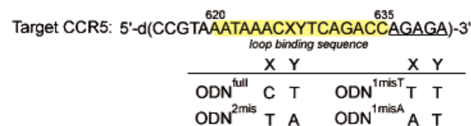


Figure 1. Illustration of (a) natural riboregulator for RpoS and (b) designed molecular-beacon-type riboregulator for luciferase switchable with DsrA and CCR5, respectively. The ribosome binding site (RBS), start codon, and target binding site are colored in red, blue, and yellow, respectively.



Translation of the template riboregulator mRNA (0.1 μg/μL or ∼1.8 pmol in 10 μL of medium) was carried out in a reconstituted prokaryotic cell-free translation system (coupled T7−transcription/translation system, Classic 1; Post Genome Institute) at 37 °C for 1 h in the presence (0.9−21.6 pmol) or absence of target ODN^{full}, followed by a chemiluminescence (CL) assay of luciferase expressed with a 96-well microplate reader (Wallac 1420). The results are shown in Figure 2a. The off-target translation activity of the present riboregulator is still substantial,[17] but the target-induced allosteric activation is rather well-behaved, showing a linear dependence on the amount of target in the range of ≤7 pmol (≤4 equiv) until saturation is reached thereafter.

The CCR5 is known as an HIV-1 co-receptor, and its sequence variations are assumed to be possible haplotype markers of acceleration/retardation of the HIV-1 disease;[18] the sequence selectivity is an important criterion here. Noncomplementary d(ACTG)$_6$AC and 2-base mismatched ODN^{2mis} (T for C and A for T at position 627 and 628, respectively) exhibit no notable enhancement with I_{on}/I_{off} = 112% (Figure 2b, lane 3) and 98%, respectively. One nucleotide selectivity depends on the identity of mismatch. ODN^{1misA} (A for C at position 627) hardly shows activation in a similar manner (I_{on}/I_{off} = 114%; Figure 2b, lane 5). On the other hand, ODN^{1misT}, which would form a relatively stable

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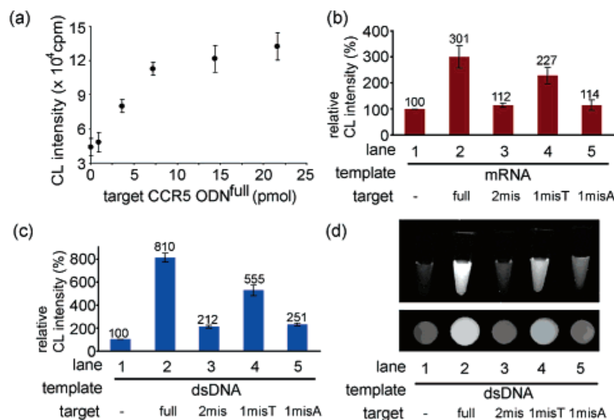

Figure 2. Chemiluminescence (CL) assay of CCR5 sequences for a 2.5 μL aliquot of translation mixture (10 μL) after treatment with 100 μL of luciferase assay solution (Promega). (a) Change in CL intensities (90 s after mixing with an assay solution, following the standard protocol) as a function of the amount of full-match target ODN^{full} with riboregulator mRNA (1.8 pmol) as probe; (b and c) relative CL intensities in the presence (18 pmol) or absence of full-match and mismatch ODN targets with mRNA (1.8 pmol) (b) or dsDNA (0.2 pmol) (c); and (d) CL images for samples in Figure 2c in vials (top) or in a 96-well plate (bottom).

GT mismatch in the resulting heteroduplex, is moderately activating (I_{on}/I_{off} = 227%; lane 4). The sensitivity or detection limit in reference to I_{on}/I_{off} can be enhanced by using a smaller amount (1 ng/μL) of riboregulator probe (to minimize target-independent translation) in combination with a sensitive luminometer (Lumat LB 9507) to 50 fmol with respect to ODN^{full} using 4.5 fmol of the probe for a 2.5 μL aliquot (I_{on} = 6362 ± 475 cpm, I_{off} = 3696 ± 42 cpm, I_{on}/I_{off} = 1.7). This detection limit is lower than that of typical fluorescence-based MBs.

As expected, the probe can also be provided in the form of DNA, from which the mRNA (riboregulator) will be transcribed in situ; it works better (Figure 2c). Under the coupled transcription/translation conditions in the same cell-free system as above, the precursor double-strand (ds) DNA[15] in a much smaller amount (0.2 pmol) gives an improved value of I_{on}/I_{off} = 810% (lanes 1 and 2) as compared with I_{on}/I_{off} = 301% when using the pretranscribed/prepurified mRNA (1.8 pmol) (Figure 2b, lanes 1 and 2).[19] The overall feature of the sequence selectivity (lanes 2−5) is similar, but in a more pronounced manner to that in the case of mRNA as the probe (Figure 2b, lanes 2−5). The selectivity can be readily appreciated using an imager (VersaDoc 3000) (Figure 2d for dsDNA and Figure S3 for mRNA) for samples in vials (top) or in a 96-well plate (bottom). An explanation for the better performance of dsDNA is to assume that the concentration of mRNA *gradually* transcribed could be kept low to make the contribution of the target-dependent translation more important. The transcription process itself is hardly affected, as expected, by the target (Figure S4).

In summary, we, for the first time, applied a luminescence-linked riboregulator system for genotyping. While allosteric factor (I_{on}/I_{off}) remains to be improved,[20] the present system possesses what are needed for good sensing, such as linearity, sensitivity (≤50 fmol of target with 4.5 fmol of mRNA probe),[21] selectivity (1 nt or at least 2 nt), and simplicity, in particular. It is indeed remarkable that sensing by chemiluminescence can be achieved with an unmodified RNA or even stabler and readily prepared/handled dsDNA; all that are needed in addition thereto are an assay kit and a cell-free translation system, both of which are commercially available. In this regard, the present method would be cell-friendly and suited for sensing in the cell,[22] where the intrinsic in-cell translation system could be directly available for a plasmid-based dsDNA probe introduced via transfection. It is also interesting to note that the output of riboregulator can be any type of enzymatic activities, thus allowing not only detection of a particular genotype but also induction of an appropriate phenotype that responds thereto in a pharmaceutical sense. Further work is now underway along these lines.

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Supporting Information Available: Figures S1−S4 (PDF) and complete ref 18. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The anti-RBS site in the MB domain is followed by a 23 nt stem-forming sequence often added to improve the stability of the transcribed RNA. The template dsDNA was prepared by stepwise insertion of the MB domain and the T7 promoter into the luciferase region of the pGL3 vector (Promega); first PCR (MB domain) with forward primer 5′-GGT CTG AAG GTT TAT TAG AAG GAG ATA TAC CAA TGG AAG ACG CCA AAA ACA TA-3′ and reverse primer 5′-TAT TCA TTA CAC GGC GAT CTT TCC G-3′ and second PCR (T7 promoter) with forward primer 5′-GAA ATT AAT ACG ACT CAC TAT AGG GAG ACC ACA ACG GTT TCC CTC TAT CTC CTG GTC TGA AGG TTT A-3′ and reverse primer 5′-TAT TCA TTA CAC GGC GAT CTT TCC G-3′. The first and the second PCR products were purified by gel electrophoresis. The riboregulator mRNA (total 1722 nt) was transcribed using MegaShortScript (Ambion) and purified by the RNeasy kit (Qiagen).
(16) The Zuker Mfold program (Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406) predicts that the loop domain forms another 5 bp stem (Figure S2).
(17) The off-target activity can be >95% suppressed by using a 28-mer ODN 5′-ATC TCC TTC TAA TAA ACC TTC AGA CCA G-3′ transacting on the stem/loop domain as an RBS sequester.
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(19) The absolute target-off chemiluminescence intensities in lane 1 of Figure 2b and that in 2c were similar to each other.
(20) The hairpin-locked structure would be generally stabilized by a longer stem and destabilized by the presence of an intrastrand anti-(anti-RBS) site and also by helicase activity, if any, in the case of in-cell sensing. Contrary to simple MBs, the present riboregulator is a big molecule, where such factors as mentioned above should be optimized as a whole to give a high level of allosteric (on/off) factor.
(21) Some codons in the template of *eukaryotic* protein luciferase turn out to be rare codons for a *prokaryotic* translation system. Codon optimization with regard to degeneracy would further improve the translation efficiency and, hence, sensitivity.
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