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Translational repression using BIV Tat peptide–TAR RNA interaction in mammalian cells†

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We developed a strategy to create novel genetically encoded switches based on translational repression. We illustrated its efficacy by incorporating two copies of an RNA hairpin in the 5′-untranslated region (UTR) of a target mRNA and demonstrating 7-fold translational repression upon expression of a ligand – the BIV Tat peptide.

Synthetic biology involves the design and construction of artificial gene circuits using well-characterized biological components, to encode novel functions or study existing networks in living cells.[1,2] Artificial gene circuits encoding diverse functions have been successfully implemented in a variety of applications.[3–6] Such circuits often consist of a number of molecular switches connected to one another in a specific configuration in order to generate a desired circuit response. Increasing the complexity of gene circuits for future applications of synthetic biology will require a larger repertoire of modular and interconnectable molecular switches with minimal crosstalk.[7,8]

A number of RNA-based molecular switches that alter gene expression in response to an input ligand have been reported.[9–13] Switches based on translational repression constitute an interesting class of RNA devices in which the translation of a target messenger RNA (mRNA) is repressed by the binding of an input ligand to sites located in the 5′-UTR of that mRNA.[14–19] Although previous studies have demonstrated the feasibility of using translational repression switches in living cells,[14–19] specific guidelines for the construction of novel switches are lacking. Moreover, most prior studies based on this approach have used small molecule ligands that need to be added exogenously.[15,17,18]

Here, we describe a design strategy to construct a novel translational repression switch, based on the well-studied interaction between the BIV Tat peptide (residues 68–81 of the BIV Tat protein) and the BIV TAR hairpin.[20,21] Our strategy was inspired by a recent report demonstrating the effect of hairpin thermodynamic stability and location in the 5′-UTR on the translational efficiency of an mRNA.[22] We demonstrated that the extent of translational repression in the presence of the BIV Tat peptide ligand depended upon the thermodynamic stability of the unbound hairpin, as calculated by Sfold (http://sfold.wadsworth.org).[23,24] We also observed greater translational repression when the hairpin was positioned close to the 5′-cap of the mRNA. Finally, the extent of translational repression could be increased significantly by the incorporation of multiple hairpin repeats in the 5′-UTR of the target mRNA.

In greater depth, it has been shown that the binding of proteins in the 5′-UTR of an mRNA can result in the repression of translation initiation.[16,25,26] Previous studies have also shown that the insertion of a hairpin, with thermodynamic stability above a certain threshold, in the 5′-UTR of an mRNA can decrease its translational efficiency significantly.[22] We reasoned that the inclusion of a hairpin in the 5′-UTR of a target mRNA (Fig. 1A), with a thermodynamic stability close to but below the critical threshold, would result in translation being ''ON'' in the absence of ligand. The binding of ligand to the hairpin would, however, increase the thermodynamic stability substantially above the critical threshold, thereby switching translation ''OFF'' in the presence of ligand (Fig. 1A).

We next decided to test this general strategy for the design of genetically encoded translational repression switches. To that end, we explored the design of switches based on the well-characterized, specific, and high affinity interaction between the BIV Tat peptide and the TAR hairpin.[21] We used a dual multiple cloning site (MCS) vector, pVITRO2-MCS-hygro, for the construction of the reporter system. GFP coding sequence was inserted in one MCS, and CFP cDNA was inserted in the second MCS (Fig. 1B). The hairpins used in this study were inserted in the 5′-UTR of GFP.

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† Electronic supplementary information (ESI) available: Materials and methods, Sfold structures, flow cytometry results, immunoblot, q-RT-PCR results, and DNA sequences. See DOI: 10.1039/c3cc43086c

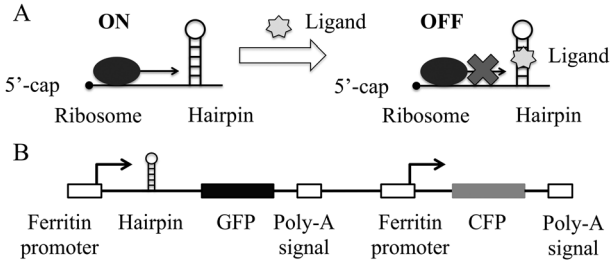

Fig. 1 Design of a switch based on translational repression. (A) Schematic indicating mechanism of translational repression. The specific binding of a ligand to a site in the 5′-UTR of an mRNA results in decreased translation. (B) Schematic for the reporter plasmid. Hairpins specific for the ligand were inserted in the 5′-UTR of GFP mRNA. CFP was expressed from another promoter on the same plasmid.

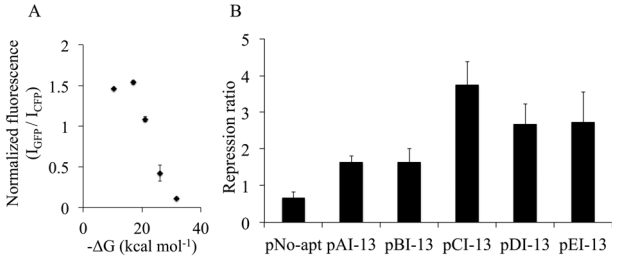

Fig. 2 Influence of thermodynamic stability of the hairpin on the extent of translational repression. (A) Influence of thermodynamic stability of the hairpin (predicted using Sfold) on the translation of GFP. 293T cells were transfected with 20 ng of reporter constructs pNo-apt, pAI-13, pBI-13, pCI-13, pDI-13, and pEI-13, containing hairpins with Sfold-predicted thermodynamic stabilities of 0 kcal mol^{-1}, −10.4 kcal mol^{-1}, −17.0 kcal mol^{-1}, −21.1 kcal mol^{-1}, −26.1 kcal mol^{-1}, and −31.8 kcal mol^{-1}, respectively. GFP and CFP fluorescence were quantified using flow cytometry. (B) Influence of thermodynamic stability of the hairpin on translational repression ratio. 293T cells were co-transfected with pCIBN-BIV Tat or pCIBN-His (80 ng), and with one of the reporter constructs pNo-apt, pAI-13, pBI-13, pCI-13, pDI-13, or pEI-13 (20 ng). Repression ratio was calculated as the ratio of the normalized fluorescence intensity (I_{GFP}/I_{CFP}) in the presence of pCIBN-His to that with pCIBN-BIV Tat. Error bars represent the standard deviation of three independent experiments.

We first determined the threshold thermodynamic stability ($\Delta G_{threshold}$), beyond which there was a significant decrease in translational efficiency. To characterize the effect of hairpin thermodynamic stability, we designed a series of hairpins with varying thermodynamic stabilities using Sfold (http://sfold.wadsworth.org).[23,24] The BIV TAR hairpin had an Sfold-predicted thermodynamic stability of −10.4 kcal mol^{-1}, significantly lower in magnitude than the mRNA translation inhibiting threshold reported by Babendure *et al.*[22] We therefore introduced additional complementary base pairs at the bottom of the BIV TAR hairpin stem to yield hairpins A, B, C, D and E with Sfold-predicted ΔG values of −10.4 kcal mol^{-1}, −17.0 kcal mol^{-1}, −21.1 kcal mol^{-1}, −26.1 kcal mol^{-1} and −31.8 kcal mol^{-1}, respectively (Fig. S1, see ESI†). These hairpins were cloned such that they were 13 nucleotides downstream of the 5′-cap. The resulting constructs were named pAI-13, pBI-13, pCI-13, pDI-13 and pEI-13, where the letters (A, B, C, D and E) denote the hairpin (Fig. S1, see ESI†), the Roman numeral (I, II or III) denotes the number of hairpin repeats, and the number after the hyphen (1, 13, or 120) denotes the position of the first hairpin downstream of the 5′-cap.

We transfected 293T cells with reporter constructs pAI-13, pBI-13, pCI-13, pDI-13, and pEI-13. We measured the intensity of GFP and CFP fluorescence using flow cytometry; the intensity of GFP fluorescence was normalized relative to that of CFP to correct for differences in transfection efficiency. As shown in Fig. 2A, we observed a significant decrease in the normalized GFP fluorescence intensity (I_{GFP}/I_{CFP}) with change in thermodynamic stability from −17 to −31.8 kcal mol^{-1}, suggesting that the threshold stability was close to −17 kcal mol^{-1}. For hairpins at a similar location, Babendure *et al.*[22] had also reported significant decreases in translational efficiency with changes in thermodynamic stability from −20 to −30 kcal mol^{-1}.

We hypothesized that binding of BIV Tat peptide to a hairpin would further increase the effective thermodynamic stability and result in translational repression. We also hypothesized that the greatest increase in translational repression would be observed for hairpins with thermodynamic stabilities close to the threshold. Since it can be difficult to express short unstructured peptides in cells, we designed a plasmid, pCIBN-BIV Tat, encoding a fusion of the BIV Tat peptide to a carrier protein CIBN (N-terminal fragment corresponding to amino acids 1–170 of plant protein *Arabidopsis* CIB1[27]). We also designed a control plasmid pCIBN-His encoding a fusion protein of CIBN to a hexa-histidine peptide.

We co-transfected 293T cells with one reporter construct (pNo-apt, pAI-13, pBI-13, pCI-13, pDI-13, or pEI-13) and with either pCIBN-BIV Tat or pCIBN-His (control). We observed greater translational repression in the presence of the CIBN-BIV Tat fusion protein as compared with the control CIBN-His fusion protein for all constructs containing the BIV TAR hairpin (Fig. 2B and Fig. S2, see ESI†). Consistent with our hypothesis, the highest translational repression ratio – the ratio of the extent of translation in the presence of CIBN-His to that in the presence of CIBN-BIV Tat – 3.6-fold (Fig. 2B), was obtained with the construct pCI-13, encoding the hairpin with an Sfold-predicted value of −21.1 kcal mol^{-1} (just above the threshold). Thus, the thermodynamic stability of the unbound hairpin may serve as a critical parameter in the design of future switches.

It has been shown that the position of a hairpin in the 5′-UTR significantly influences its ability to affect translational efficiency.[22] We hypothesized that the position of the hairpin C in the 5′-UTR could also play an important role in determining the extent of translational repression. To examine the effect of hairpin location, we made additional constructs pCI-1 and pCI-120 (Fig. 3A), where the hairpin C was located 1 and 120 nucleotides downstream of the 5′-cap, respectively. We co-transfected 293T cells with pCIBN-BIV Tat or pCIBN-His, and with plasmids encoding one of the three reporter constructs that differed in the position of the hairpin C in the 5′-UTR (Fig. 3A).

We observed similar translational repression ratios for pCI-1 and pCI-13 (Fig. 3B). In comparison, the reporter construct pCI-120 showed no repression in the presence of BIV Tat peptide as compared with the control peptide (Fig. 3B and Fig. S3, see ESI†). These results confirm that a cap-proximal location of the hairpin greatly facilitates translational repression.

Next, we investigated whether increasing the number of repeats of hairpin C in the 5′-UTR, would influence the extent

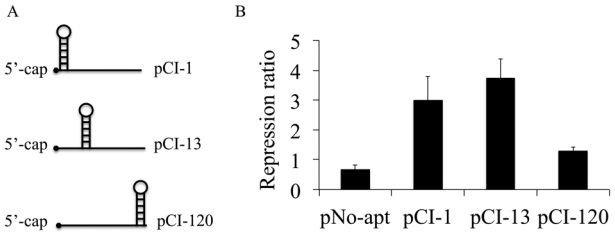

Fig. 3 Cap proximal location of the hairpin is necessary for translational repression. (A) Schematic showing the position of hairpin C in the 5′-UTR. (B) Influence of the position of hairpin C on translational repression ratio. 293T cells were co-transfected with pCIBN-BIV Tat or pCIBN-His (80 ng), and with reporter constructs pCI-1, pCI-13, or pCI-120 (20 ng). Fluorescence was determined using flow cytometry and the repression ratio was calculated as the ratio of fluorescence intensities of GFP and CFP in the presence of pCIBN-His to that in the presence of pCIBN-BIV Tat. Error bars represent the standard deviation of three independent experiments.

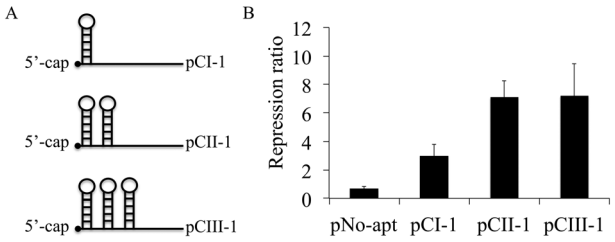

Fig. 4 Increasing the number of repeats of a hairpin in the 5′-UTR significantly increases translational repression ratio. (A) Schematic for the reporter constructs pCI-1, pCII-1 and pCIII-1 containing different number of repeats of hairpin C. (B) Influence of the number of repeats of hairpins on the repression ratio. 293T cells were co-transfected with reporter constructs pCI-1, pCII-1 or pCIII-1 (20 ng) and pCIBN-BIV Tat or pCIBN-His (80 ng). GFP and CFP fluorescence was determined using flow cytometry. The repression ratio was calculated as the ratio of GFP/CFP value in the presence of pCIBN-His to that in the presence of pCIBN-BIV Tat. Error bars represent the standard deviation of three independent experiments.

of repression. To test this hypothesis, we used three constructs – pCI-1, pCII-1, and pCIII-1 – consisting one, two, and three copies of hairpin C in tandem, starting 1 nucleotide downstream of the 5′-cap in the 5′-UTR of GFP reporter mRNA (Fig. 4A). We co-transfected 293T cells with one of these reporter constructs and pCIBN-BIV Tat or pCIBN-His. The repression ratio for pCI-1 was ~3-fold and that for pCII-1 and pCIII-1 was ~7-fold (Fig. 4B and Fig. S4, see ESI†). This result is consistent with previous studies where a significant improvement in the translational repression ratio was observed by the insertion of additional hairpins in the 5′-UTR.[15,18] The repression ratio of ~7-fold also lies within the range (5–16 fold) reported in previous studies.[14–16] We used immunoblotting to confirm that the observed decrease in GFP fluorescence intensity was indeed due to a decrease in the amount of GFP protein (Fig. S5, see ESI†). We also quantified the cytoplasmic GFP mRNA levels using q-RT-PCR and found them to be similar for samples co-transfected with either pCIBN-BIV Tat or pCIBN-His (Fig. S6, see ESI†). These results confirmed that the control of gene expression was at the translational level.

To summarize, we have constructed a novel translational repression switch, pCII-1, which gives a translational repression ratio of ~7-fold upon the expression of CIBN-BIV Tat. In designing this switch, we observed that a hairpin with a thermodynamic stability of −21.1 kcal mol^{-1} offered the best translational repression ratio. We also observed that the position of the hairpin in the 5′-UTR was an important parameter, with the translational repression ratio being highest for cap-proximal hairpins. Lastly, we observed significant increases in the translational repression ratio when two hairpins were placed in tandem in the 5′-UTR. We note that Systematic Evolution of Ligands by Exponential Enrichment (SELEX) can be used to identify a wide variety of ligand–aptamer pairs with high affinity and selectivity.[28,29] Application of the design strategy used in the present study to other ligand–aptamer pairs may thus guide the design of a large repertoire of translational repression switches with minimal crosstalk.

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