

Custom design of metabolism

James C Liao

Two new approaches, RNA-enzyme fusions immobilized onto a surface and artificial RNA switches, could transform metabolic pathway engineering.

Metabolic engineering requires both robust approaches to predicting and testing the outcomes of possible pathway manipulations and reliable control mechanisms to subsequently allow the fine tuning of those pathways. Two papers, one recently published in *Science*¹ and the other in this issue², report progress on both fronts. In the first, Jung and Stephanopoulos¹ use DNA molecules to capture different RNA-enzyme fusions from a specific biochemical pathway in a 96-well microtiter plate assay. By altering the levels of the capture DNA molecules to control the exact amounts of each of the enzymes involved, the authors optimize, in a series of iterative experiments, the quantity of each enzymatic component of the pathway that results in the highest protein yield. In a related paper, Isaacs *et al.*² develop a series of RNA switches (riboswitches) that enable precise control of target gene translation by blocking the ribosome binding site (RBS) of the gene of interest. By introducing different combinations of riboswitches, precise network manipulations should be possible. Taken together, these two complimentary approaches offer new possibilities at both ends of the metabolic engineering spectrum.

Cellular metabolism is regulated by multiple layers of control at the levels of transcription, mRNA degradation, post-translational modification and kinetics. The control loops at the first two levels regulate the amount of proteins expressed in the cell, whereas the last two tiers modify their activities. Such a multi-level regulatory network makes efforts to redesign metabolism difficult. When a single

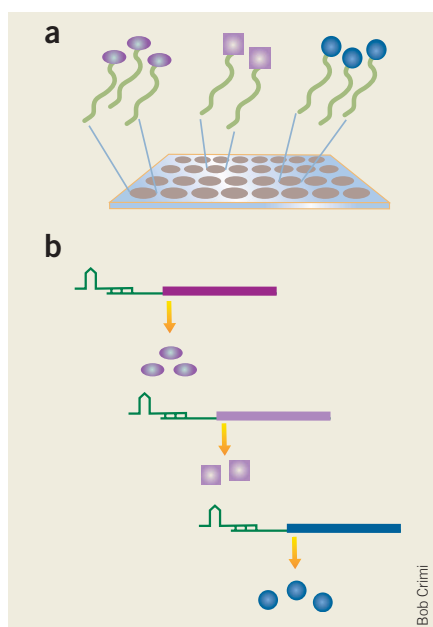


Figure 1 Two approaches for gaining control of metabolism. (a,b) To manipulate a hypothetical pathway (a), the metabolism-on-a-chip technique (b) determines the optimal ratios of enzyme levels in the pathway through versatile and quantitative immobilization of enzymes. (c) With artificial riboswitches, *in vivo* control of gene expression can be achieved following RNA hybridization to block and unblock ribosome binding sites.

Jung and Stephanopoulos applied the puromycin-assisted mRNA-protein fusion technique⁵ to perform ‘metabolism-on-a-chip,’ which allows fast optimization of enzyme levels in a metabolic pathway or network. The mRNA-protein fusions can be achieved through parallel *in vitro* translation. The resulting mRNA-enzyme fusions are subsequently captured by single-stranded DNA on a 96-well microtiter plate (Fig. 1). The authors show that the amount of enzyme immobilized can be quantitatively determined by the amount of capture DNA used and that the DNA-RNA hybridization is specific to the individual mRNA-enzyme fusions. When the enzymes in a metabolic pathway are all fused to their respective mRNA and immobilized, the resulting system is a metabolic-network-on-a-chip, the enzyme levels of which can be easily changed by the amount of capture DNA.

enzyme in a pathway is artificially overexpressed, the pathway flux often remains largely the same, even after potential problems in expression, protein folding or post-translational modification are solved. This phenomenon is attributed to kinetic regulation through complex interactions between small metabolites and enzymes, such as an overexpressed enzyme being counterbalanced by a buildup of inhibitors. In this case, flux control is shared by multiple enzymes through the balancing of different branches or different steps in the same branch. Further improvement requires avoiding inhibitor buildup, providing cosubstrates, or recycling cofactors by tuning expression levels of other proteins in the network^{3,4}. Such a balancing act is nontrivial and is difficult to predict quantitatively. However, this problem is fundamental to the understanding and engineering of metabolic networks.

The authors use this platform to study the trehalose biosynthesis pathway, which is relatively simple, but has a loop providing the cosubstrate UDP-glucose from glucose 6-phosphate. The results clearly show that by increasing a single enzyme level, the overall flux reaches a plateau after an initial increase. The key to increasing the flux is maintaining the ratio between phosphoglucomutase (Pgm) and trehalose 6-phosphate synthase (OstA). The importance of balancing enzyme levels in a pathway has been demonstrated before^{3,4}. The advantage of the *in vitro* system

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presented here is the quantitative and evolutionary nature of the approach to pathway optimization. Because the *in vitro* translation and mRNA-protein fusion techniques are generalizable, this approach is applicable to any system. With the knowledge of optimal enzyme ratios, we now can design expression systems *in vivo* to implement the strategy.

On the other hand, metabolism is dynamic. Cells modulate metabolism through gene expression controls to adapt intracellular and extracellular states. Consequently, the optimal expression strategy should be dynamically adjusted. Traditionally, deregulation has been a strategy to overcome the cellular response to metabolic perturbation. Recently, synthetic gene regulatory circuits have been introduced to reprogram cellular transcription^{6,7}, metabolism⁸ and communication^{9,10}. These gene circuits use components of regulatory machinery in the cell, but employ an artificial connectivity to achieve a designed function. Although synthetic gene circuits have enjoyed impressive success, further progress is limited by the components available for circuit design.

In this issue, Isaacs *et al.* report a new strategy for designing synthetic gene circuits using artificial riboswitches. These custom-designed noncoding RNA sequences (*cis*-repressed mRNA) fold back to bind with the RBS necessary for prokaryotic translation, and thereby inhibit translation. The stem-loop blocking the RBS is opened by a *trans*-activating RNA that hybridizes with the leading part of the *cis*-repressed mRNA. Thus, the *trans*-activating RNA, whose expression can be controlled by an inducible promoter, such as the arabinose promoter, serves as an activator of the gene coded by the mRNA.

The success of artificial riboswitches provides additional evidence that RNA molecules can serve as regulatory elements. This mode of regulation significantly expands the capability of synthetic gene circuits. First, multiple artificial riboswitches can be designed and coexist in the same cell, and each can be linked to any gene of interest. Second, the *trans*-activating RNA acts as a positive regulator, which is less common in prokaryotes. Third, when working with a native promoter, the artificial riboswitch can modulate the expression level near the physiological state. This capability is useful when attenuation of expression is desirable in a metabolic process. Finally, this mode of regulation can serve as a model to identify native riboswitches in the genome.

The 'metabolism-on-a-chip' and artificial riboswitch platforms represent important analytical approaches to metabolic engineering and synthetic gene circuits. Reconstituted *in vitro* systems have played an important role

in defining minimal components required for a given function. In contrast to the traditional reconstituted systems, the metabolism-on-a-chip technique can deal with larger systems, and allows independent analysis of regulatory components, particularly small molecules, which are difficult to manipulate *in vivo*. It is expected to provide control targets for *in vivo* engineering of cell function. Final implementation of control can then be achieved using artificial riboswitches in addition to existing regulatory components. These two recent advances promise to bring the custom-design of metabolism to a new level.

The ins and outs of gene expression control

Francine B Perler

Temperature-sensitive mutant inteins inserted within two transcription factors provide a new tool for controlling gene expression in yeast and flies.

Temperature-sensitive mutants have been a mainstay of genetic analysis since the early 20th century. But often such mutants can be difficult to isolate for a protein of interest and, if found, the amino acid changes introduced by the mutation may compromise function. In this issue, Zeidler *et al.*¹ combine temperature-sensitive (TS) inteins—protein elements capable of self-excision from a precursor protein and ligation of the flanking protein regions²—with the yeast transcription factors Gal4 and Gal80 to create a system that overcomes these problems. For cells containing genes under the control of the Gal4 promoter, a simple temperature shift activates protein splicing of hybrid Gal4 and Gal80, resulting in excision of the intein and restoration of transcription factor functionality. The approach enables gene expression to be turned on or off in a temporal, spatial and cell type-specific manner in both single-celled and multicellular organisms.

Intein-mediated protein splicing is a post-translational, autocatalytic reaction requiring no known host cell cofactors, making inteins very portable. However, each intein is more or less sensitive to proximal host protein

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sequences, which are the substrate of the intein enzymatic activity^{2–4}. Over 170 inteins have been catalogued in InBase, the intein database⁵. In naturally occurring precursors, protein splicing is usually required for host protein activity. However, when inserting inteins into new sites, host protein activity in the precursor and the spliced product should be tested using inactive and active inteins, respectively. The positional effects of protein splicing in foreign contexts can often be overcome by trying several intein insertion sites. Zeidler *et al.* have eliminated this problem by inserting temperature-dependent splicing mutants of the *Saccharomyces cerevisiae* vacuolar ATPase subunit (VMA) intein into transcriptional regulators that control expression of essentially any target protein.

The transcriptional regulators they chose to modify were Gal4 and Gal80. Gal4 is a widely used enhancer in many transgenic organisms, especially *Drosophila melanogaster*, where almost 7,000 lines have been catalogued in GETDB (Gal4 Enhancer Trap Insertion Database)⁶. Gal80 binds to Gal4 and blocks the latter's ability to activate transcription. Zeidler *et al.* demonstrated intein-mediated conditional target gene expression in yeast, *D. melanogaster* tissue culture cells and in transgenic *D. melanogaster* imaginal discs (cell cluster progenitors of adult organs). This method is readily transferable to the multitude of genes currently regulated by Gal4.

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