stood, so it has been difficult to overcome this problem. HSV-TK presents an additional problem because it is expressed from an internal promoter in testis and kills spermatids; therefore, only females transmit transgenes containing HSV-TK[6].

Taking advantage of the fact that mice are at least 1,000-fold more resistant to DT than are primates, Saito *et al.* used transgenic techniques to target the human DT receptor to mouse hepatocytes and show that the transgenic mice developed severe hepatotoxicity in response to doses of DT that had no effect on wild-type mice.

DT is made by *Corynebacterium diphtheriae* as a single polypeptide that is proteolytically cleaved into a two-chain protein. The larger B-subunit binds to membrane receptors and is then internalized. Within acidic endosomes, the smaller A-chain dissociates and passes through the membrane into the cytoplasm, where it becomes catalytically active and ADP-ribosylates elongation factor 2, thereby inactivating protein synthesis.

The human DT receptor was discovered about 10 years ago by genetic transfer of toxin sensitivity from human to mouse cells[7]. It encodes a 208-amino-acid, single-pass transmembrane protein also known as HB-EGF. Diphtheria toxin binds to the extracellular domain of HB-EGF near the membrane, in a region that in the mouse and primate differs by only a few amino acids[8]. The DT receptor contains a three-loop EGF-like motif also found in transforming growth factor alpha. The extracellular EGF-like domain can be proteolytically released, possibly by the same transmembrane metalloproteinase (ADAM17, also known as TACE, or the related ADAM9) that releases transforming growth factor alpha[9].

The authors of the paper in this issue used the albumin promoter and enhancer to direct expression of HB-EGF to hepatocytes. Treating the mice with 500 ng DT per kg body weight resulted in serum markers of hepatotoxicity and death of the mice in two to three days. By lowering the dose to 50 ng/kg, the authors achieved transient heptotoxicity and the mice recovered. In contrast, wild-type mice showed no adverse effects after receiving DT at a dose of 50,000 ng/kg. The authors detected HB-EGF mRNA expression in five of the six transgenic founders produced, and the sensitivity to DT was roughly proportional to the level of expression. With this approach, a differential response can be achieved depending on either the level of transgene expression or the DT dose used.

This HB-EGF gene seems to offer substantial advantages over previous conditional toxigene strategies. Although other systems have suffered from transgene silencing, a high percentage of the transgenic mice described by the authors expressed the gene appropriately—perhaps because HB-EGF is of mammalian origin. In addition, there is a huge window of DT effectiveness, with no apparent side effects in wild-type mice.

The authors have not yet tried other transgenes, so it is too soon to know how general the technique may be. The HB-EGF transgene driven by cell-specific promoters could result in EGF-like biological activity in some contexts, but it might be possible to inactivate the EGF-like domain without destroying DT binding, if necessary. Tagging human HB-EGF with green fluorescent protein could facilitate the visualization of cells expressing it and their demise after DT treatment.

Transgenic mice expressing human HB-EGF could be a useful model for transplantation studies because wild-type donor cells survive a DT challenge. This application would be similar to that of transgenic mice expressing urokinase that manifest chronic heptocellular degeneration; as a consequence, transplanted hepatocytes from wild-type mice, or even rats, were used to reconstitute a normal liver[10]. Unlike urokinase toxicity, DT-mediated toxicity may be conferred to any cell type by judicious targeting of HB-EGF expression, thereby providing a selective advantage to transplanted wild-type stem cells that would be resistant to DT.

HB-EGF should prove to be a powerful tool for exploring many other biological problems in mice, and there is no apparent reason it could not be applied to other organisms that are resistant to DT.

1. Saito, M. *et al*. *Nat. Biotechnol*. **19**, 746–750 (2001).
2. Palmiter, R.D. *et al*. *Cell* **50**, 435–443 (1987).
3. Behringer, R.R. *et al*. *Genes Dev*. **2**, 453–461 (1988).
4. Hara, J. *et al*. *Neuron* **30**, 345–354 (2001).
5. Borrelli, E. *et al*. *Proc. Natl. Acad. Sci. USA* **85**, 7572–7576 (1988).
6. Braun, R.E. *et al*. *Biol. Reprod*. **43**, 684–693 (1990).
7. Naglich, J.G. *et al*. *Cell* **69**, 1051–1061 (1992).
8. Louie, G.V. *et al*. *Mol. Cell* **1**, 67–78 (1997)
9. Peschon, J.J. *et al*. *Science* **282**, 1279–1280 (1998).
10. Rhim, J.A. *et al*. *Science* **263**, 1149–1152 (1994).

A PURE approach to constructive biology

In vitro protein synthesis using a mixture of purified components now offers the possibility of efficient production of labile products and more detailed study of the translation process.

James Swartz

Protein synthesis is one of the most complicated basic biological processes. In this issue, Shimizu *et al.*[1] convincingly demonstrate that even this complex metabolic system can be reconstructed from its purified component parts—more than 100 in all. To reconstitute highly active protein synthesis, the researchers used a purified mixture of 46 tRNAs and individually purified another 32 catalysts and cofactors. Their approach, which they term the "PURE system," provides a new way to produce proteins, opens new avenues for the study of translation, and expands the concept of metabolic engineering.

Figure 1 shows the main components of the PURE system. Transcription and translation are coupled and driven by the high-energy phosphate bonds of creatine phosphate (CrP) and nucleoside triphosphates. First, T7 RNA polymerase transcribes the template DNA into mRNA. Because the system is essentially free of nucleases, the mRNA provides a stable template for translation. Before amino acids can be polymerized in the proper order, they must first be activated by the appropriate tRNAs in reactions catalyzed by 20 different aminoacyl–tRNA synthetases. The reactions shown in green constitute the ATP regeneration system, in which creatine phosphate regenerates the ATP required for tRNA amino-acylation. Because the acylation reaction hydrolyzes ATP to AMP, two creatine phosphates are required for each acylation. An adenylate kinase activity (in this case, provided by myokinase) allows ATP to convert AMP to ADP so that creatine kinase can regenerate the ATP. The pyrophosphate side product (PP_i) has a relatively high affinity for Mg^{2+} and, if allowed to accumulate, would inhibit the various nucleoside phosphate reactions by sequestering Mg^{2+}. Pyrophosphatase is added to hydrolyze the PP_i to phosphate (reaction not shown), which has a much lower affinity for Mg^{2+}.

In the GTP regeneration system (shown in

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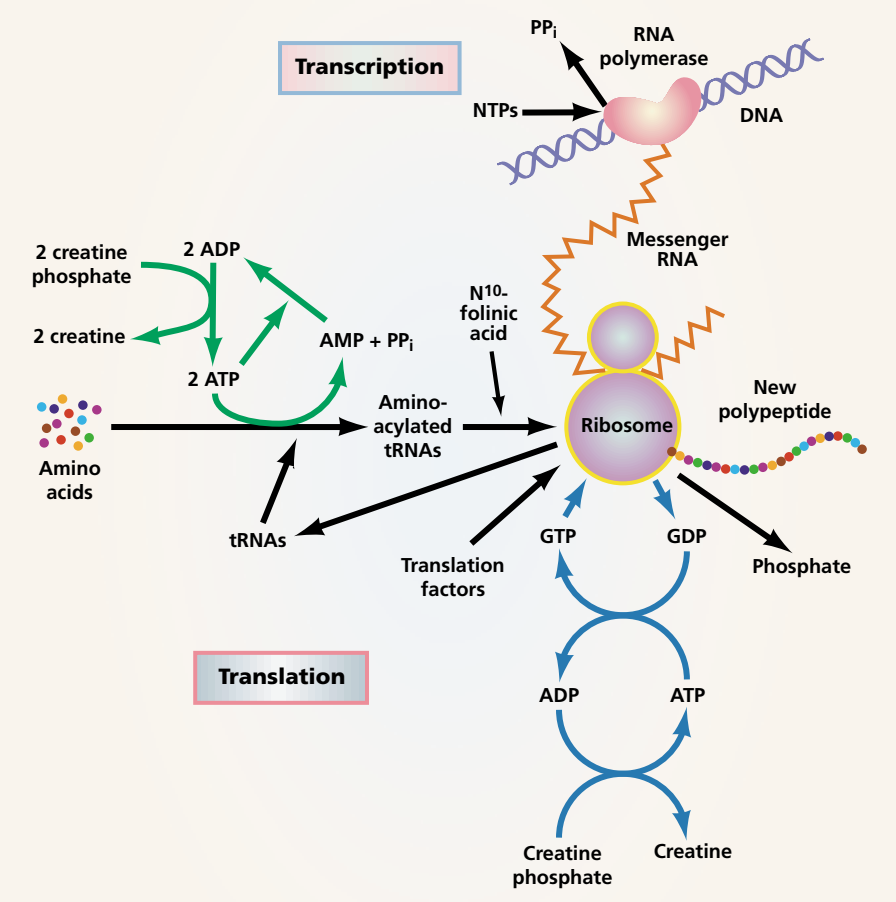

Figure 1. The PURE system. More than 100 purified catalysts, cofactors and reactants are needed to activate protein synthesis. The high-energy phosphate bonds of creatine phosphate drive the ATP regeneration system (green) and the GTP regeneration system (blue).

blue), creatine phosphate regenerates the GTP needed for high-fidelity amino acid polymerization. To initiate translation, N^{10}-folinic acid formylates the Met-$tRNA_f$ in a reaction catalyzed by methionyl-tRNA transformylase. The various translation factors and tRNAs are recycled and, in that sense, are catalytic. Although more than 100 factors are needed, the final translation reaction is relatively simple:

Amino acids + CrP + N^{10}-folinic acid →
Polypeptide + Cr + P_i + Tetrahydrofolate

Clearly, the PURE system is highly active. It produces 170 µg/ml of protein per hour from 10 mM creatine phosphate and 6 mM nucleoside triphosphates. Assuming that four to five ATPs are required for each peptide bond[2], the conversion efficiency is 39–48% of the theoretical maximum (based on the energy sources). The PURE synthetic rate is about 1.8 peptide bonds per ribosome per second (averaged over one hour) compared with a rate of approximately 20 *in vivo*[3]. Even though the PURE rate is lower, it is still quite good for an *in vitro* system. Rates of 10 codons per second were measured with a semi-purified cell-free system, but these were maintained for only a few minutes.[4] The PURE system sets an impressive new standard for productivity using purified components. Nonetheless, the current rate and efficiency indicate that there are still opportunities for substantial improvement, as the authors note.

It is also interesting to compare the performance of the PURE system to that of more traditional cell-free protein synthesis using crude *Escherichia coli* cell extracts. Shimizu *et al.* point out that the system of Kim *et al.*[5] was more productive (400 µg/ml), although the energy requirement was also higher (50 mM creatine phosphate). Recently, my group has shown that energy-conversion yields similar to those of the PURE system can be obtained using crude cell extracts.[6] We have also observed energy efficiencies >70% and yields of 800 µg/ml from 30 mM phosphoenol pyruvate (J.S., unpublished data). Such high efficiencies are surprising: as Shimizu *et al.* mention, cell extracts rapidly and unproductively hydrolyze compounds with high-energy phosphate bonds[1,7]. The most likely explanation is that protein synthesis reactions have a higher affinity for ATP than do the degenerative reactions.

In the recently reported cell-extract system[6], energy is obtained from both phosphoenol pyruvate and its direct reaction product, pyruvate. As the pyruvate is generated from both the productive and degenerative reactions, it provides a relatively slow, continuous, and reliable supply of ATP. ATP measurements from my laboratory indicate ATP concentrations in the 100–200 µM range compared with 3–5 mM in the *E. coli* cytoplasm. The micromolar ATP concentrations are adequate for protein synthesis but may be too low for substantial flux to competing reactions.

Although the PURE system may not be more energy efficient than cell-extract systems, it does offer tremendous flexibility and precision in adjusting concentrations of the various components. As the authors point out, this could be a significant advantage for the incorporation of unnatural amino acids at stop codons. The absence of nucleases and proteases will also be useful for synthesizing labile oligopeptides or for techniques such as ribosome display[8]. However, such advantages come at a cost. Approximately 36 fermentations and purifications are needed to provide the reagents of the PURE system. Each must be accompanied by a certain degree of assessment and quality control. Although the proteins chosen for demonstration—for example, dihydrofolate reductase—folded properly, it is likely that chaperones and foldases will also be necessary for more robust folding and more complex proteins.

Moreover, since metabolic pathways such as glycolysis can be beneficial[6], additional factors may be necessary to optimize the PURE system. It is not apparent at what point the flexibility and predictability afforded by the PURE system become too costly. Undoubtedly, this will depend not only on the specific application but also on how well crude cell extracts can be "tamed" to work efficiently and predictably. Nevertheless, it is obvious that the PURE system represents a substantial breakthrough in our ability to reconstitute complex biological processes and to study them thoroughly as integrated units.

1. Shimizu *et al. Nat. Biotechnol.* **19**, 751–755 (2001).
2. Ehrenberg, M. *et al. J. Mol. Biol* **211**, 739–749 (1990).
3. Bremmer, H. & Dennis, P.P. in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (eds. Neidhardt, F.C. *et al.*) 1553–1569 (ASM Press, Washington, DC, 1996).
4. Pavlov, M.Y. and Ehrenberg, M. ARchives Biochem.Biophys. 328, 9–16 (1996).
5. Kim, D.-M. *et al. Eur. J. Biochem.* **239**, 881–886 (1996).
6. Kim, D.-M. & Swartz, J.R. *Biotechnol. Bioeng.* **71**, 309–316 (2001).
7. Kim, D.-M. & Swartz, J.R. *Biotechnol. Prog.* **16**, 385–390 (2000).
8. Schaffitzel, C. *et al. J. Immunol. Meth.* **231**, 119–135 (1999).