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Single Protein Production in Living Cells Facilitated by an mRNA Interferase

Technique

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Summary

We designed a single-protein production (SPP) system in living *E. coli* cells that exploits the unique properties of MazF, a bacterial toxin that is an ssRNA- and ACA-specific endoribonuclease. In effect, MazF functions as an "mRNA interferase," because it efficiently and selectively degrades all cellular mRNAs in vivo, resulting in a precipitous drop in total protein synthesis. Concomitant expression of MazF and a target gene engineered to encode an ACA-less mRNA results in sustained and high-level (up to 90%) target expression in the virtual absence of background cellular protein synthesis. Remarkably, target synthesis continues for at least 4 days, indicating that cells retain transcriptional and translational competence despite their growth arrest. SPP technology works well for *E. coli* (soluble and membrane), yeast, and human proteins. This expression system enables unparalleled signal to noise ratios that should dramatically simplify structural and functional studies of previously intractable but biologically important proteins.

Introduction

Most bacteria contain suicidal genes whose expression leads to growth arrest and eventual death upon exposure to cellular stress (Engelberg-Kulka and Glaser, 1999; Engelberg-Kulka et al., 2004). These toxin genes are usually coexpressed with their cognate antitoxin genes in the same operon (referred to as an addiction module or antitoxin-toxin system). The *E. coli* chromosome contains five addiction modules (Christensen et al., 2003), of these, the MazE-MazF system is the most extensively characterized. The X-ray structure of the MazE-MazF complex (Kamada et al., 2003) is known, and the precise enzymatic activity of MazF has been recently determined (Zhang et al., 2004b; Zhang et al., 2003).

MazF is a sequence-specific endoribonuclease that exclusively cleaves ssRNAs at ACA sequences (Zhang et al., 2004b; Zhang et al., 2003). We refer to MazF as an mRNA interferase because its primary target is mRNA in vivo. In contrast, cellular tRNAs appear to be protected from cleavage because of their extensive secondary structure, whereas rRNAs appear to evade degradation by MazF because of their close association with ribosomal proteins. Therefore, MazF expression results in nearly complete degradation of mRNA, leading to severe reduction of protein synthesis in conjunction with growth arrest (Zhang et al., 2003). Proteins with sequence similarity to MazF are found in a number of bacteria or on their extrachromosomal plasmids. Recently, an R100 plasmid-encoded relative in *E. coli* called PemK was also shown to be a sequence-specific endoribonuclease that possesses broader cleavage specificity than MazF (Zhang et al., 2004a).

In the present study, we have exploited the unique cleavage properties of MazF to design an SPP system in living *E. coli* cells. Upon induction of a gene engineered to express an ACA-less mRNA (without alteration of its amino acid sequence), high levels of individual target protein synthesis were sustained for at least 96 hr in the virtual absence of background protein synthesis. Therefore, the toxic effect of MazF is directed at mRNA with minimal side effects on cellular physiology. In fact, despite their state of growth arrest, these cells retain essential metabolic and biosynthetic activities for energy metabolism (ATP production), amino acid, nucleotide biosynthesis, and transcription/translation. In addition to demonstrating the efficacy of the SPP system for bacterial, yeast, and human proteins, the technology was also effective for overexpression of an integral inner membrane protein whose natural levels of expression are relatively low. The SPP system is a powerful protein expression technology that, in addition to being a highly effective method for production of recombinant proteins, yields unprecedented signal to noise ratios when new protein synthesis is monitored by isotopic labeling. Therefore, this technology may enable structural and functional studies of proteins in intact, living cells using nuclear magnetic resonance (NMR).

Results

MazF Induction Leads to a Dramatic Reduction in Protein Synthesis

Induction of MazF expression mediated by IPTG from a low copy number plasmid containing a T7-inducible promoter (pACYC*mazF*) resulted in growth arrest in *E. coli* BL21(DE3) cells (data not shown). To better understand the mechanism of this growth arrest, our earlier work focused on investigation of the enzymatic activity of MazF. Although we previously demonstrated that cellular protein synthesis was dramatically reduced at 37°C as a consequence of the efficient and site-specific cleavage of mRNA by MazF, we first wanted to test if cell growth arrest (and the concomitant reduction in protein synthesis) also occurred when MazF was induced at the cold-shock temperature of 15°C. If so, we might be able to design a protein expression system that combined the attributes derived from both a low cellular temperature and MazF expression. Exposure of *E. coli* cells to low temperatures initiates the cold-

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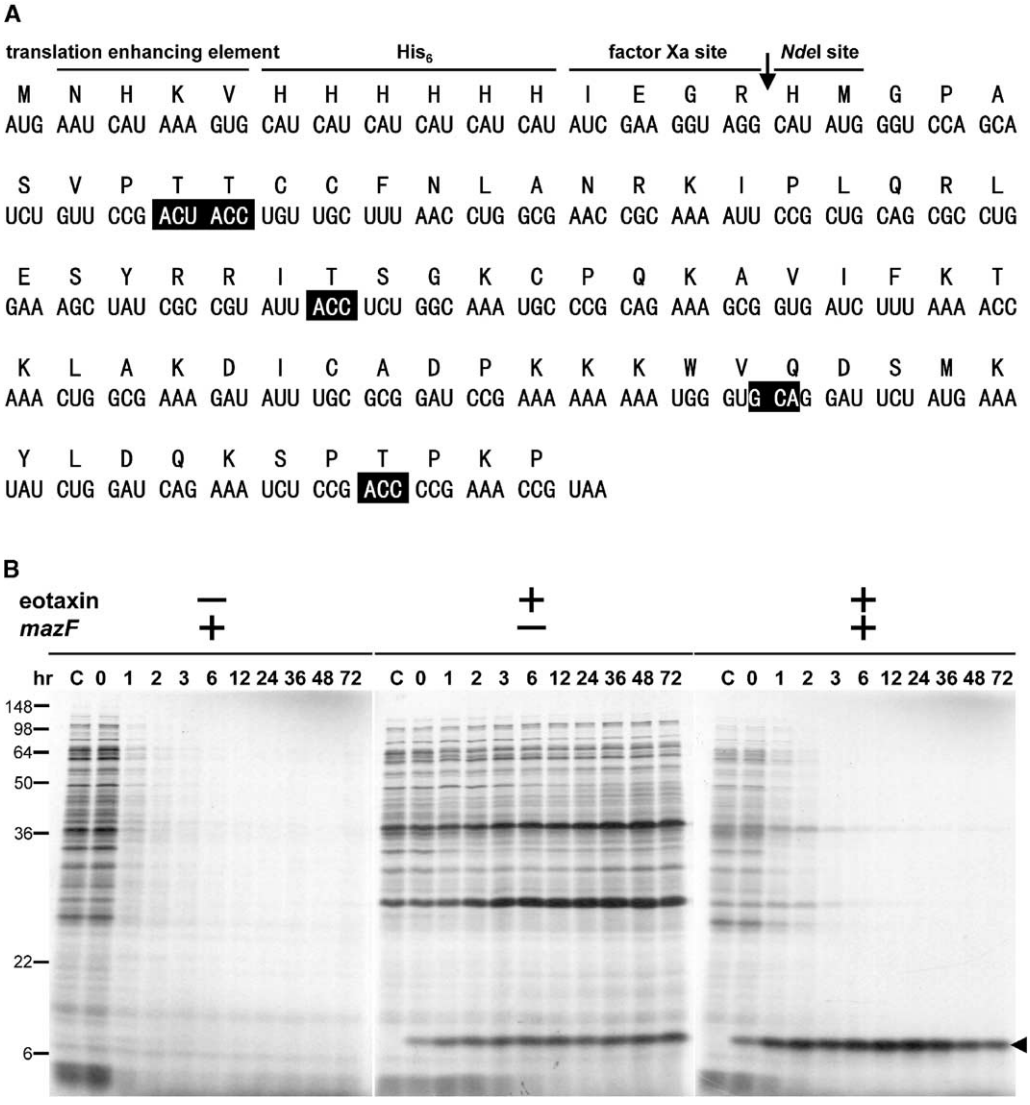

Figure 1. Expression of Human Eotaxin in *E. coli* with and without MazF Coexpression

(A) The amino acid sequence of the human eotaxin fusion. The sequence below it represents that of the mRNA derived from the synthetic eotaxin gene (designed with preferred *E. coli* codons). Mature, proteolytically processed eotaxin comprises 74 residues from G18 (shown here right after the NdeI site) from P91. Triplets highlighted in black were changed to ACA for the experiment shown in the left panel of Figure 2C.

(B) The effect of MazF expression on eotaxin production. Upon reaching an OD_{600} of 0.5, cultures of *E. coli* BL21(DE3) transformed with pACYC*mazF* and pColdI(SP-1)eotaxin (right), pACYC*mazF* alone (left), or pColdI(SP-1)eotaxin alone (middle) were shifted from 37°C to 15°C for 45 min. New protein synthesis was then monitored by isotopic labeling with [^{35}S]-methionine for 15 min before (C control lane) or at intervals after (0–72 hr) IPTG induction. Equivalent amounts of cell lysate, derived from equal culture volumes, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Molecular weight markers on the left; the position of eotaxin designated by an arrow to the right.

shock response (Thieringer et al., 1998) believed to enhance both the solubility and/or stability of proteins (Qing et al., 2004). If we created a cellular milieu optimal for protein solubility and stability where background protein synthesis was also extremely low, we could then express proteins inherently resistant to MazF-mediated degradation from genes engineered to yield ACA-less mRNA transcripts.

First, to determine how MazF expression affects cell growth and protein synthesis during cold-shock conditions, cells were initially incubated for 45 min at 15°C followed by IPTG induction at 15°C of MazF (Figure 1B, left). Cells were then pulse labeled with [^{35}S]methionine for 15 min at time intervals up to 72 hr. In addition to the immediate cessation of cell growth upon IPTG induction of MazF (not shown), total cellular protein synthesis was dramatically inhibited 1 hr after induction; after 6 hr, the synthesis of cellular proteins was dramatically diminished. This extremely low level of background synthesis was sustained up to 72 hr postinduction. Therefore, as at the normal *E. coli* growth temperature of 37°C, MazF expression under cold-shock conditions leads to both rapid growth arrest and the severe reduction of total protein synthesis.

Stable Expression of a Human ACA-less mRNA in MazF-Induced Cells

Once we had established the suitability of cold-shock conditions for the SPP technology, we tested the next crucial component of the system, stable expression of a target protein. We speculated that an mRNA devoid of ACA sequences is likely to be stably maintained in the cells expressing toxin. Therefore, coexpression of a target protein could ideally take place in cells with minimal or no cellular protein synthesis.

To test this possibility we synthesized an altered gene for human eotaxin (Garcia-Zepeda et al., 1996)—a chemokine of high commercial and potential therapeutic value—that encoded an ACA-less mRNA for the mature form of the 74 amino acid residue protein (Figure 1A). Design of this synthetic gene was based only on the amino acid sequence of eotaxin, not from the native human eotaxin gene (Hein et al., 1997). Codon selection of the synthetic gene was primarily influenced by the need for an ACA-less mRNA transcript, *E. coli* codon usage preferences were then applied (Nakamura et al., 2000). In fact, codon degeneracy is pivotal for the success of the MazF-based SPP system. The ACA triplet can be altered to other cleavage-resistant sequences without changing the protein amino acid sequence, regardless of its position in the reading frame. For example, ACA in the +1 reading frame (threonine) can be altered to AC**C**, AC**G**, or AC**U** (altered bases are in bold); when ACA falls in the +2 reading frame, all permutations of XAC-A can be changed to X**A**U-A (where X is any of the four bases); similarly, XXA-CA can be changed to XX**G**-CA when ACA is in the +3 reading frame with a single exception, AUA-CA. In this case the sequence is simply altered to AUU-CA or AUC-CA to retain the isoleucine codon.

The synthetic eotaxin gene was first cloned into pColdI(SP-1) and pColdI(SP-2), modified versions of the pColdI vector designed for high yield protein expression under cold-shock conditions (Qing et al., 2004). The resulting constructs, pColdI(SP-1)eotaxin and pColdI(SP-2)eotaxin, respectively, were transformed into the *E. coli* BL21(DE3) strain routinely used for T7 promoter expression studies (the plasmid that expresses MazF has an IPTG-inducible T7 promoter). Insertion of a target gene at the NdeI site of either vector results in its fusion to a 17 residue amino terminal sequence comprising four components: (1) the translation-enhancing element from the *cspA* gene (encoding the major cold-shock protein CspA) that supports high-level protein expression upon cold shock, (2) a six histidine residue tag sequence, (3) a factor Xa cleavage site, and (4) the histidine-methionine sequence derived from the NdeI site, a restriction site that enables the in-frame insertion of any target gene. The modifications of pColdI(SP-1) and pColdI(SP-2) involved removal of ACA sequences to help stabilize the fusion protein in the presence of MazF. In pColdI(SP-1), two ACA sequences were converted to AUA (one between the Shine-Dalgarno sequence and the initiation codon and the second in the translation-enhancing element right after the initiation codon). pColdI(SP-2) was then derived from pColdI(SP-1), and three additional ACA sequences in the 5′ untranslated region (UTR) were altered to MazF resistant sequences by base substitutions (to GCA, AUA, and GCA from the 5′ ACA to the 3′ ACA, respectively).

After the cells containing pColdI(SP-1)eotaxin alone were cold shocked at 15°C and acclimated to the low temperature for 45 min, IPTG was added to induce eotaxin production. Subsequent pulse labeling with [^{35}S]methionine for 15 min (Figure 1B, middle) demonstrated that there was sustained, high-level synthesis of eotaxin from the onset of induction up until the last 72 hr time point along with a spectrum of other cellular proteins. Quantification of the level of labeled eotaxin relative to total labeled protein at the 12 hr time point revealed that eotaxin represented ~11% of total cellular protein.

When both eotaxin and *mazF* genes were coexpressed in *E. coli* BL21(DE3) harboring both pACYC-*mazF* and pColdI(SP-1)eotaxin, background cellular protein synthesis was dramatically reduced after a 3 hr induction, whereas robust levels of eotaxin production continued for 72 hr (Figure 1B, right). Interestingly, the level of eotaxin production in this experiment was higher (Figure 1B, right; 47% of total protein production at 12 hr) than that in the absence of MazF induction (Figure 1B, middle; 11% at 12 hr). We attribute this ~5-fold enrichment to increased ribosome availability for eotaxin mRNA translation, because at this point, the majority of cellular mRNAs should have been degraded by MazF.

The impact of removal of ACA sequences from the 5′ UTR of the eotaxin expression vector was demonstrated in Figure 2A. Induction of pACYC*mazF* and pColdI(SP-2)eotaxin revealed both a striking enrichment of eotaxin over background protein synthesis and overall levels of eotaxin production substantially higher than that with pColdI(SP-1)eotaxin. Background cellular protein synthesis diminished sooner than that with pColdI(SP-1)eotaxin; no distinct background cellular protein bands were discernible after the 12 hr time point. In fact, ~90% of [^{35}S]methionine was incorporated into eotaxin 12 hr after MazF induction. Remarkably, the high level of eotaxin production did not diminish even after 96 hr of induction.

In addition to monitoring new protein synthesis after MazF induction, we documented total cellular protein levels by Coomassie blue staining (Figure 2B). Although the eotaxin band was very faint initially, eotaxin appeared to be the most abundant cellular protein 12 hr after MazF induction. Eotaxin production then peaked at 24 hr, suggesting that there is a threshold level of eotaxin production (~14% of total cellular protein) in MazF-induced cells. Eotaxin produced at 24 hr was mostly in the soluble fraction after low-speed centrifugation (not shown). Because [^{35}S]methionine incorporation was sustained for 96 hr (Figure 2A), it appears that eotaxin production and degradation in the SPP system equilibrates after 24 hr.

Finally, we examined if eotaxin production is influenced by nutrient conditions. Protein expression profiles (as assessed by Coomassie blue staining) were equivalent in cells grown in either rich LB medium or minimal M9 medium (not shown). Therefore, protein production by the SPP system does not appear to fluctuate with nutrient availability. Because M9 medium lacks preformed amino acids, this result also indicates

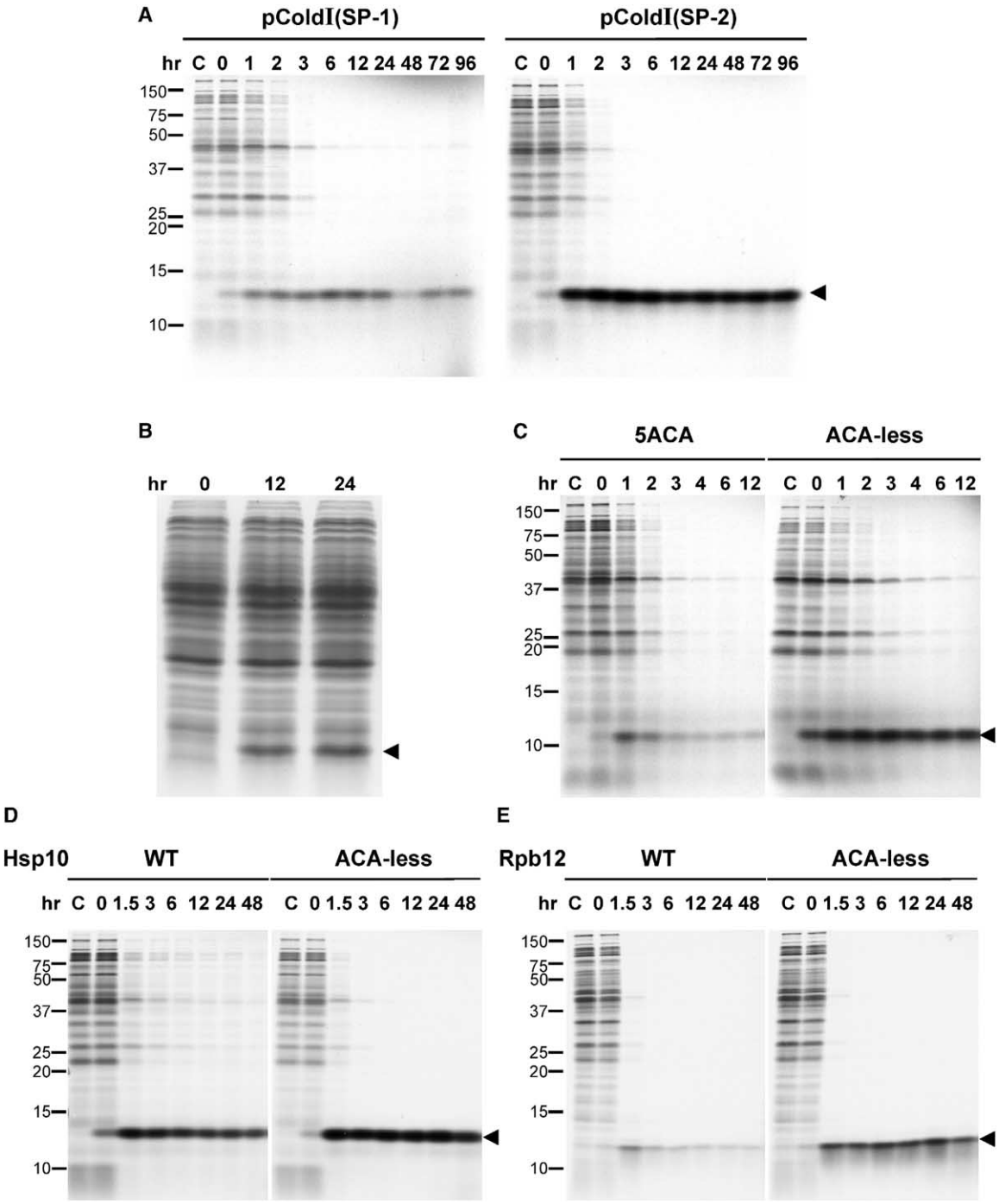

Figure 2. Effect of ACA Sequences on Expression of Human Eotaxin and Yeast Proteins

(A) Cultures of *E. coli* BL21(DE3) transformed with pACYC*mazF* and either pColdI(SP-1)eotaxin (left) or pColdI(SP-2)eotaxin (right) were shifted from 37°C to 15°C for 45 min. New protein synthesis was then monitored by isotopic labeling with [^{35}S]-methionine for 15 min before (C control lane) or at intervals after (0–96 hr) IPTG induction. Equivalent amounts of cell lysate, derived from equal culture volumes, were subjected to SDS-PAGE followed by autoradiography. Molecular weight markers for both panels are shown on the left.

(B) Cells from an *E. coli* BL21(DE3) culture carrying pACYC*mazF* and pColdI(SP-2)eotaxin were harvested after cold shock and IPTG induction at the times indicated followed by SDS-PAGE and Coomassie blue staining. Equivalent amounts of cell lysate, derived from equal culture volumes, were loaded. The position of eotaxin in both panels is designated by the arrow to the right.

(C)eotaxin genes without (right) and with five (left) ACA-encoding sequences were expressed in the pColdI(SP-1) vector in BL21(DE3) cells subjected to the same conditions described for Figure 1. The positions of the new ACA sequences are shown in Figure 1A. Molecular weight markers are on the left; the position of eotaxin designated by an arrow is to the right.

(D and E) Wild-type (wt) and ACA-less yeast Hsp10 (D) and Rpb12 (E) mRNAs were expressed from pColdI(SP-2) along with wt MazF from pACYC*mazF*. Experiments were carried out as described for Figure 1. Molecular weight markers are on the left; the position of each target protein is designated by an arrow to the right.

that amino acid biosynthetic pathways are functional. In fact, although they are growth arrested (OD_{600} levels remain constant after MazF induction; data not shown), MazF-induced cells clearly retain the full spectrum of biosynthetic functions necessary to support mRNA transcription and translation. These biosynthetic functions include (1) energy metabolism; (2) amino acid and nucleotide biosynthesis; (3) RNA polymerase, ribosome, and tRNA activity; and (4) the presence of essential accessory factors that facilitate these processes. MazF induction does not markedly enhance degradation of existing proteins visible by Coomassie staining (Figure 2B), so the majority of amino acids for translation are likely derived from biosynthetic pathways.

ACA Sequences Destabilize Protein Production in MazF-Induced Cells

In order to test the effect of ACA sequences on the stability of the target protein in MazF-induced cells, we altered the ACA content of either eotaxin or MazF mRNAs. In the case of eotaxin, we now added up to five ACAs (human eotaxin mRNA normally has one ACA) without altering the protein sequence; for MazF, we removed all nine ACAs without amino acid changes. First, an eotaxin gene was engineered to produce an mRNA containing five ACA sequences (Figure 1A). When this gene was expressed in pColdI(SP-1) along with pACYC*mazF*, markedly lower levels of eotaxin production were now observed (Figure 2C, left) compared to ACA-less eotaxin (Figure 2C, right). This low level of eotaxin synthesis further declined to background levels. These results indicate that ACA sequences in target protein mRNAs reduce the amount of protein that is subsequently translated, consistent with the cleavage specificity of MazF for mRNA.

Curiously, MazF mRNA has an unusually high ACA content (111 residues, 9 ACAs). Therefore, MazF may negatively regulate its own activity. If so, we would expect wild-type (wt) MazF to be less potent in our SPP expression system than a form derived from an ACA-less mRNA. Although cells overexpressing the ACA-less MazF grew a little more slowly than those overexpressing wt MazF, the signal to noise ratio was further improved with the ACA-less MazF without significant effects on the target protein production (not shown). Taken together, the results of the ACA addition and subtraction experiments clearly demonstrate that ACA sequences in mRNAs play the crucial role in protein production in MazF-induced cells.

Application of the SPP System to Yeast Proteins

Once the SPP system was developed and optimized with eotaxin, we wanted to test its efficacy for high-level expression of bacterial cytoplasmic and membrane proteins as well as other eukaryotic proteins. We first applied the technology to two yeast proteins—the heat shock factor Hsp10 and RNA polymerase subunit Rpb12 (Figures 2D and 2E, respectively). The native genes for Hsp10 and Rpb12 encode mRNAs with three or one ACA(s), respectively. Surprisingly, wt Hsp10 was expressed and translated at a reasonably high level (Figure 2D). However, when all three ACA sequences were removed, considerable enhancement of Hsp10 synthesis was observed (at 24 hr, ACA-less Hsp10 represented 84% of total [^{35}S]-methionine incorporation in contrast to 50% with wt Hsp10), and background protein synthesis was significantly reduced.

Although wt Rpb12 mRNA contains only one ACA, low Rpb12 and cellular background protein levels were observed at all time points. However, expression levels of ACA-less Rpb12 were significantly higher than wt Rpb12, whereas background protein synthesis remained low (Figure 2E). Our observation that mRNAs containing only one ACA (Rpb12) are not necessarily translated to higher levels than those with three ACAs (Hsp10) suggest that mRNA sensitivity to MazF cleavage is not dictated by ACA content alone but may rely on the position of the ACA in a single-stranded region of an mRNA or the efficiency of translation of an mRNA by ribosomes. Both Hsp10 and Rpb12 proteins expressed by the SPP system were soluble (not shown).

Application of the SPP System to *E. coli* Proteins

Next, we applied the SPP technology to two *E. coli* cytoplasmic proteins, EnvZB and CspA. EnvZB comprises the 161 residue ATP binding domain of EnvZ, an osmosensing histidine kinase. CspA is a 70 residue major cold-shock protein. Expression of both wt and ACA-less EnvZB or CspA was examined for up to 96 hr and visualized by SDS-gel electrophoresis followed by Coomassie blue staining (Figures 3A and 3B). The amount of ACA-less EnvZB steadily increased during the 96 hr incubation, reaching 17% of the total cellular protein, whereas the amount of wt EnvZB was 11% at 96 hr. Wt CspA gene expression resulted in CspA protein levels representing 16% of the total cellular protein 96 hr post-induction. However, CspA production nearly doubled—to 28% of the total cellular protein—from the ACA-less CspA gene after the same amount of time. As with human eotaxin (Figure 2B), no significant changes in the number and abundance of cellular proteins (present from 0 hr to 96 hr) were noted upon extended expression of CspA or EnvZB. As with eotaxin, Hsp10, and Rpb12 in the SPP system, EnvZB and CspA were also soluble (not shown).

Application of the SPP System to an Integral Membrane Protein

Expression of recombinant membrane proteins in *E. coli* and other expression systems remains a formidable challenge (Grisshammer and Tate, 1995). To determine the flexibility of the SPP system, we tested its efficacy for expression of an *E. coli* membrane protein referred to as either signal peptidase II, lipoprotein signal peptidase, or LspA. LspA (164 amino acids) is specifically required for cleavage of the signal peptides of lipoproteins (Tokuda and Matsuyama, 2004) and is considered to be a very low abundance integral inner membrane protein with four predicted transmembrane domains. Wt LspA mRNA contains three ACAs; an *lspA* gene encoding an ACA-less mRNA was constructed and cloned into pColdIV(SP-2), a vector identical to pColdI(SP-2) that lacks the translation-enhancing element, His_6 tag, and factor Xa cleavage site. Thus, unlike the other target proteins, LspA was not expressed as a

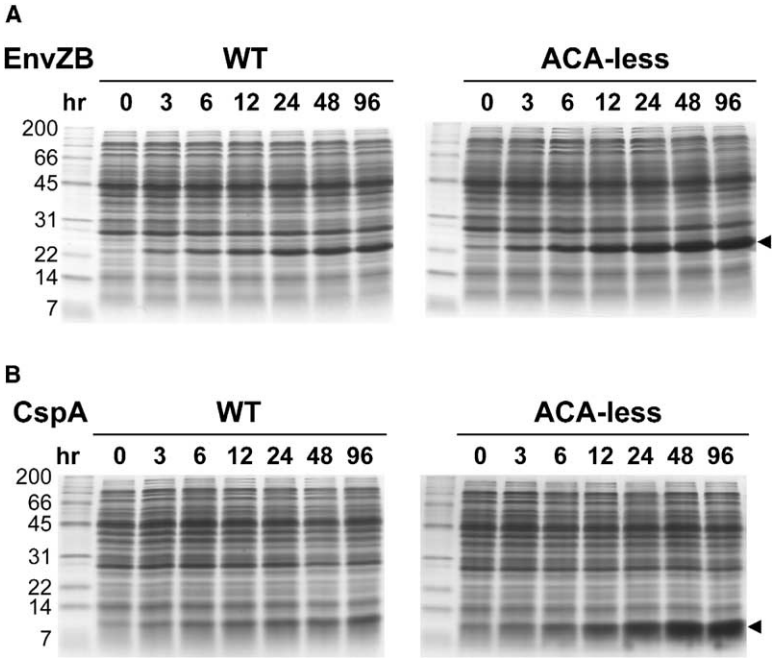

Figure 3. Expression of *E. coli* Proteins in the SPP System

Wt and ACA-less EnvZB (A) and CspA (B) mRNAs were expressed from pColdI(SP-2) along with wt MazF from pACYC*mazF*. Expression was carried out as described for Figure 1, and experiments were followed by SDS-PAGE and Coomassie blue staining. Molecular weight markers are on the left; the position of each target protein is designated by an arrow to the right.

fusion protein in order to preclude potential problems with its localization to the inner membrane.

Although coexpression of LspA and ACA-less MazF was apparently toxic to the cells—levels of total [^{35}S] methionine incorporation sharply declined only 1 hr after IPTG induction of *mazF*(–9ACA)—sufficient incorporation in LspA was achieved early on, and background protein synthesis was minimal at the 1 hr time point (Figure 4A). Unlike any of the other soluble proteins shown above, maximal rates of [^{35}S]methionine incorporation into LspA were observed at the 0 hr time point, immediately after induction of LspA and MazF(–9ACA). This indicates that overproduction of LspA is highly toxic so that the rates of [^{35}S]methionine incorporation into LspA were reduced as soon as its production was induced. Subsequent ultracentrifugation to enrich for membrane proteins helped to improve the signal to noise ratio, especially at the 0 hr time point (Figure 4B). Finally, we were able to demonstrate that LspA synthesized by the SPP system was correctly localized to the inner membrane fraction as demonstrated by its solubilization in 0.5% sarkosyl (Figure 4C). Therefore, the SPP system can be applied for the expression of either soluble or membrane proteins.

Discussion

We applied the distinctive enzymatic properties of the bacterial toxin MazF to develop and effectively implement a protein expression technology that yields signal

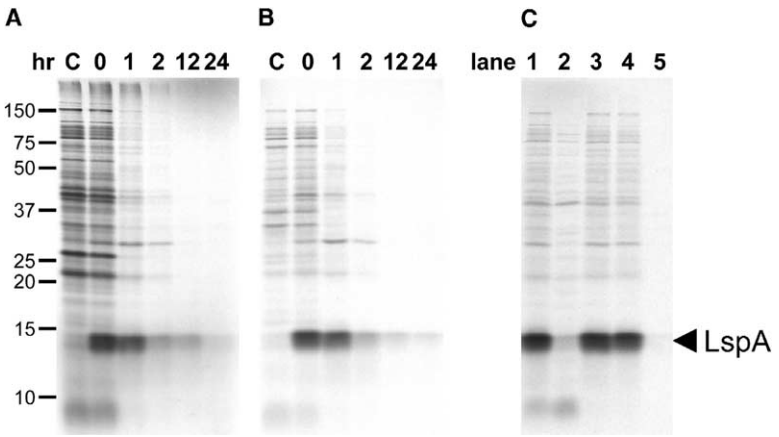

Figure 4. Expression of the Inner Membrane Protein LspA with the SPP System

(A) LspA expression in pColdIV(SP-2) relative to total cell protein synthesis was carried out as described in Figure 1 by using pACYC-*mazF*(–9ACA). Molecular weight markers for all three panels are shown on the left.

(B) LspA expression was profiled as in (A) but only membrane fractions were loaded for each time point.

(C) Analysis of the membrane fraction components. The membrane fraction from (B) was further fractionated after LspA was induced for 1 hr to separate the inner from the outer membrane fractions. Lane 1, total cellular proteins; lane 2, the soluble fraction obtained after ultracentrifugation; lane 3, the membrane fraction obtained after ultracentrifugation; lane 4, sarkosyl soluble fraction (inner membrane fraction); and lane 5, sarkosyl insoluble fraction (outer membrane fraction). The position of LspA in all three panels is indicated by the arrow.

to noise ratios unrivaled by other in vivo expression systems (Yokoyama, 2003). We have discovered that *E. coli* cells can be easily reprogrammed to manufacture astonishingly high levels of virtually pure target protein (~90% of new protein synthesis). Intrinsic to the success of this system is the extended state (at least 96 hr at 15°C) of what we refer to as "quasi-dormancy" exhibited by *E. coli* host cells. MazF induction does not appear to interfere with pathways for generation of ATP or the biosynthesis of nucleotides and amino acids, all of which are required for RNA and protein synthesis. The existence of this altered state of suspended animation was illuminated only when we attempted to exploit a loophole—inducing synthesis of a gene encoding an ACA-less mRNA—in cells programmed for arrested growth followed by eventual cell death. It is unclear when these quasi-dormant cells reach a point where they resist resuscitation.

Bacterial viability is often determined by the ability of cells to form colonies upon plating. Studies on the viability of *E. coli* cells after MazF induction revealed that cells could be revived by coexpression of the antitoxin/antidote MazE for a limited window of time (Pedersen et al., 2002). Although the effect of MazF can be reversible, there may be a threshold after which these compromised cells are destined to die. Curiously, because the *mazE* mRNA contains two ACAs, enlistment of a MazF resistant derivative of MazE would likely widen the window of time for recovery of colony-forming units. The molecular details that distinguish MazF-induced quasi-dormancy from phenomena such as the quiescent state of cells in stationary phase or the dormant state of spores will require further exploration.

We have exploited this quasi-dormant state to establish an expression system in vivo. Cells were fully capable of persistent and high-level protein synthesis from ACA-less mRNAs. There are numerous advantages of the SPP system over the best bacterial expression systems. First, SPP technology represents a dramatic improvement of our recently developed pCold system (Qing et al., 2004), yielding signal to noise ratios unmatched by any in vivo expression system. pCold vectors support enhanced protein expression and stability relative to the popular pET-based systems because they enlist the CspA promoter/UTR to achieve high-level expression at 15°C (the low temperature improves protein solubility and stability). The SPP system builds on these advantages by virtually eliminating background cellular protein synthesis.

Second, the SPP technology is particularly suitable for structural analysis of proteins by either NMR or X-ray crystallography, because nearly exclusive isotopic labeling of the target protein—^{15}N and ^{13}C for NMR or selenomethionine for X-ray—can be achieved. Recently, we have shown that NMR structural determination of a protein can be accomplished by using cell lysates without protein purification by expressing a protein of interest by high-expression, cold-shock vectors, pCold (Qing et al., 2004).

Third, SPP technology enables not only cell-lysate NMR but also NMR structural studies of proteins in their natural environment. "In-cell NMR spectroscopy" (Dedmon et al., 2002; Serber and Dotsch, 2001; Serber et al., 2001a; Serber et al., 2001b) is the only way to learn about the actual structures and dynamics of proteins inside of living cells under truly physiological conditions. The experimental approaches taken so far are, however, absolutely dependent upon the overproduction of a target protein. Because the SPP system can eliminate almost all background protein synthesis during isotope enrichment, it is anticipated that it will enable NMR structural studies on proteins in the living cell under physiological conditions.

Additionally, our SPP system enables NMR structural studies of proteins that are toxic or tend to easily aggregate when expressed at high levels. Lower levels of net protein production can be tolerated without sacrificing purity because of the high signal to noise ratio afforded by the system as shown with LspA, a very minor inner membrane protein.

Another significant advantage of the SPP system is that protein production can be carried out in a highly condensed culture. This attribute translates into significant cost savings for NMR or X-ray analysis of protein structures. Additionally, the SPP system can be exploited as a living bioreactor for biosynthesis of non-protein components.

Finally, SPP technology represents a desirable alternative to cell-free translation systems and the standard yeast *Pichia pastoris* heterologous protein expression system. Commercially available bacterial and wheat germ in vitro expression systems are much more expensive than the SPP system. Alternatively, protocols for their preparation are elaborate and labor intensive (Kigawa et al., 2004; Kigawa et al., 2002; Morita et al., 2003; Sawasaki et al., 2002). Although *P. pastoris* can reach high cell densities and target protein expression can be >30% of total soluble protein, it does not rival the signal to noise ratio achieved by SPP technology, especially upon isotopic labeling for NMR structure applications (Cereghino et al., 2002; Cereghino and Cregg, 2000; Houard et al., 2002).

Even the best bacterial system cannot overcome the caveat that applies to eukaryotic proteins expressed in *E. coli*—the success rate for recovery of natively folded proteins diminishes incrementally for proteins derived from increasingly complex eukaryotes. Yeast and mammalian SPP systems are currently in development to enable expression of a broad spectrum of eukaryotic proteins for structural, functional, and therapeutic applications.

Experimental Procedures

Strains and Plasmids

E. coli BL21(DE3) cells were used for expression studies. The *mazF* gene was cloned into the NdeI-XhoI sites of pACYCDuet (Novagen) to create plasmid pACYC*mazF*. pACYC*mazF*(–9ACA) was constructed by site-directed mutagenesis using pACYC*mazF* as template. The eotaxin gene was synthesized on the basis of the optimal *E. coli* codon usage (see Figure 1A) and cloned into the NdeI-HindIII sites of pColdI(SP-1) to create plasmid pColdI(SP-1)eotaxin. pColdI(SP-1) was constructed from pColdI (Qing et al., 2004) as described in the text by altering two ACA sequences (one between the Shine-Dalgarno sequence and the initiation codon, the other in the translation-enhancing element) with pColdI (eotaxin) as template. Mutagenesis was carried out by using *Pfu* DNA polymerase (Stratagene) based on the protocol for the QuikChange Site-Directed Mutagenesis Kit (Stratagene) or by two-step PCR (Ho et

al., 1989). pColdI(SP-2) was constructed from pColdI(SP-1) by converting the three ACA sequences in the 5′ UTR to GCA (5′ ACA), AUA (middle ACA), and GCA (3′ ACA). pColdI(SP-1)eotaxin(+ACA) was constructed from pColdI(SP-1)eotaxin by modifying the eotaxin gene to encode an mRNA containing five ACAs at the positions highlighted in Figure 1A. The wt yeast *hsp10* gene was amplified by PCR from yeast genomic DNA and cloned into the NdeI-BamHI sites of pColdI(SP-2) to create plasmid pColdI(SP-2) Hsp10. The ACA-less *hsp10* gene was created by two-step PCR using the wt *hsp10* gene as template and cloned into the NdeI-BamHI sites of pColdI(SP-2) to create plasmid pColdI(SP-2) Hsp10(–ACA). The *hsp10* ORF, comprising 106 codons, contains three ACA sequences (GCA-CAA for A25-Q26, ACA for T29, and CCA-CAG for P76-Q77) that were converted to GC**C**-CAA, AC**C**, and CC**C**-CAG, respectively (altered bases are in bold). The wt and ACA-less *rpb12* gene was amplified by PCR from a wt Rpb12 plasmid and cloned into the NdeI-BamHI sites of pColdI(SP-2) to create plasmid pColdI(SP-2)Rpb12 and pColdI(SP-2)Rpb12(–ACA), respectively. The *rpb12* ORF, comprising 70 codons, contains one ACA for T10 that was converted to AC**C**. The ACA-less *envZB* gene was created by two-step PCR using the wt *envZB* gene as template and cloned into the NdeI-BamHI sites of pColdI(SP-2) to create plasmid pColdI(SP-2)*envZB*(–ACA). The *envZB* ORF, comprising 161 codons, contains three ACA sequences (GAA-CAA for E92-Q93, GAC-AGU for D105-S106, and ACA for T158) that were converted to GA**G**-CAA, GA**U**-AGU, and AC**C**, respectively. The ACA-less *cspA* gene was created by two-step PCR using the wt *cspA* gene as template and cloned into the NdeI-BamHI sites of pColdI(SP-2) to create plasmid pColdI(SP-2)*cspA*(–ACA). The *cspA* ORF, comprising 70 codons, contains three ACA sequences (GAC-AAA for D15-K16, GUA-CAC for V32-H33, and UAC-AAA for Y42-K43) that were converted to GA**U**-AAA, GU**G**-CAC, and UA**U**-AAA, respectively. The ACA-less *lspA* gene was generated by two-step PCR and cloned into the NdeI-BamHI sites of pColdIV(SP-2) to create plasmid pColdIV(SP-2)*lspA*(–ACA). The *lspA* ORF, comprising 164 codons, contains three ACA sequences: T8 was converted from ACA to AC**C**, N97-N98 from AAC-AAU to AA**U**-AAU, and K163-Q164 from AAA-CAA to AA**G**-CAA.

Assays of Protein Synthesis In Vivo

E. coli BL21(DE3) carrying one or two of the plasmids described above was grown in M9-glucose medium at 37°C. When the OD_{600} reached 0.5, the culture was shifted to 15°C for 45 min to acclimate the cells to cold-shock conditions. 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to induce expression of the downstream gene (MazF or target gene). At time intervals, 1 ml of the culture was added to a test tube containing 10 μCi [^{35}S]-methionine. After isotopic labeling for 15 min at 15°C, 0.2 ml of 40 mg/ml methionine was added and incubated for 5 min. The labeled cells were washed with M9-glucose medium and the pellets resuspended in 100 μl of SDS-PAGE loading buffer. 10 μl of each sample was analyzed by SDS-PAGE followed by autoradiography.

Preparation of the Membrane Fraction

The cells harvested from 1 ml of the culture by centrifugation (10,000 × g for 5 min) were suspended in the 50 mM Tris-HCl (pH 7.5) and disrupted by sonication. The total membrane fraction was obtained by ultracentrifugation (100,000 × g, for 60 min) after the removal of intact cells. The inner membrane fraction was solubilized from the total membrane fraction by using 0.5% sarkosyl (Filip et al., 1973).

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