

## CHAPTER

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# The Role of Cell-Free Rabbit Reticulocyte Expression Systems in Functional Proteomics

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### Abstract

In the broad area of functional proteomics, that is the global characterization of proteins and their function, cell-free rabbit reticulocyte lysate (RRL) has been used extensively to elucidate the mechanisms of mammalian translation, cotranslational modifications, post-translational modifications and translocation of proteins. More recently, RRL has been used as the workhorse for manufacturing the proteins engaged in interaction, selection and protein evolution studies from DNA or mRNA libraries either in microarray, display or in vitro expression cloning (IVEC) technologies. This chapter highlights recent functional proteomics applications that use cell-free mammalian RRL.

### Abbreviations

CMM, canine microsomal membrane; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; GPI, glycosylphosphatidylinositol; IVC, in vitro compartmentalization; IVEC, in vitro expression cloning; PCR, polymerase chain reaction; PTT, protein truncation test; RRL, rabbit reticulocyte lysate; RT-PCR, reverse transcription-polymerase chain reaction; SP cells, semipermeabilized cells.

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### Introduction

In late 1950s and early 1960s researchers first demonstrated that radioactive amino acids could be incorporated into hemoglobin in cell-free rabbit reticulocyte lysate (RRL).<sup>1,2</sup> Since then RRL has been used to elucidate the highly complex events that encompass translation, from initiation to termination.<sup>3-5</sup> RRL has also proven useful in understanding cotranslational folding of nascent polypeptide chains, protein targeting and post-translational folding. During the 1970s, researchers showed that RRL could be manipulated for exogenously directed mRNA protein synthesis, so that only a single protein of interest was synthesized.<sup>6</sup> In the 1990s, the development of coupled transcription/translation, in which RRL is supplemented with T7, T3, or SP6 RNA polymerases further simplified the expression of protein targets.<sup>7,8</sup> DNA-directed protein synthesis in RRL has some advantages over mRNA-primed RRL including the elimination of mRNA handling, and it usually achieves higher levels of protein synthesis. An advantage of cell-free RRL over other cell-free systems (*E. coli* or wheat germ extracts) is that the mammalian environment more

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closely mimics human cells. Cell-free RRL is generated from lysed reticulocytes isolated from phenylhydrazine-treated rabbits. The lysed reticulocytes are treated with micrococcal nuclease to remove endogenous mRNA. The RRL is optimized and supplemented with components that give optimal translation when priming with mRNA and, in the case of a coupled system, optimal translation/transcription for priming with DNA that contains the appropriate RNA phage polymerase promoter sequences.

Cell-free RRL has the same advantages as all cell-free systems over cell-based expression systems: substantial time-savings (two hours versus 24–48 hours for protein expression), the ability to adapt to high-throughput formats, increased tolerance to additives and less sensitivity to toxic or proteolytic proteins. The current use of cell-free RRL is substantial as illustrated by a HighWire Press® search covering January 2000 to April 2006 that yielded more than 3,000 articles containing the phrase “rabbit reticulocyte.” This chapter will focus on recent applications that use RRL for cell-free functional proteomics.

## Membrane Topology

### *Synthesis of Membrane Proteins*

Membrane protein topology is often described based on the predicted amino acid sequence and algorithms that estimate hydrophobicity and probable secondary structure of a stretch of amino acids.<sup>9</sup> However, different algorithms or different stringencies applied to the same algorithm can predict different topologies, and many algorithms fail to account for cotranslational processing events or the effects of post-translational modifications on protein topology.<sup>9</sup> Translation in a cell-free system containing microsomes or semipermeabilized cells can provide empirical data about membrane protein topology.

Using a prepared lysate supplemented with endoplasmic reticulum (ER)-derived microsomal membranes such as canine pancreatic microsomal membranes (CMMs)<sup>10-12</sup> or digitonin-permeabilized cells (semipermeabilized cells),<sup>13</sup> membrane proteins can be successfully synthesized, translocated and modified *in vitro*. Semipermeabilized (SP) cells have some advantages over ER-derived microsomes. SP cells are more likely to contain the necessary components for the correct folding and modification of proteins normally expressed by the cells, and they have a spatially intact ER and Golgi system that better approximates the cellular environment.<sup>13</sup> Additionally, SP cells can be more efficient at specialized modifications such as adding glycosylphosphatidylinositol (GPI) anchors.<sup>14</sup> Using SP cells and RRL without dithiothreitol allows disulfide bond formation and efficient folding of proteins such as MHC class I heavy chain.<sup>15</sup>

Proteins can be associated with the membrane in a variety of ways. Integral membrane proteins span both leaflets of the phospholipid bilayer with one or more alpha helical transmembrane domains consisting of approximately 20 hydrophobic amino acids. Peripheral membrane proteins can be associated with a single membrane leaflet either by means of a fatty acid modification, such as prenylation, myristoylation, or a GPI anchor, or by association of a predominantly hydrophobic stretch of amino acids. Cell-free translation systems such as RRL supplemented with microsomes or SP cells can provide information about how a particular protein associates with a membrane. For instance, treating isolated microsomes with sodium carbonate dissociates peripheral but not integral membrane proteins from microsomes or semipermeabilized cells.<sup>16</sup> Such treatment has been used to show that the glycoprotein GP4 produced by the equine arteritis virus is an integral membrane protein, while the GP3 protein, produced by the same virus, is membrane-anchored.<sup>17</sup>

### *Protease Protection Assays*

Protease protection assays are often used to help determine membrane protein topology and orientation. Water-soluble proteases cannot freely cross the lipid bilayer of microsomes, so segments of proteins that are in the lumen of the microsomes will not be subject to protease digestion unless the microsomes are first permeabilized. Assuming that the lumen of microsomes represents the

lumen of the ER, the segments of proteins in the lumen of the microsomes become extracellular once a protein is inserted into the plasma membrane.

Several studies have used protease protection assays to determine the topology of membrane proteins, including determining which of two proposed topologies is correct for cytochrome  $b_5$ .<sup>18</sup> In this study, failure of carboxypeptidase Y to remove C-terminal labeled methionines of cytochrome  $b_5$  suggests that the C-terminus is inside the lumen of the ER and inaccessible to the protease.<sup>18</sup> Often, the protease assay is performed on proteins translated in the presence of microsomes or SP cells. The microsomes are incubated with the protease with or without concomitant detergent solubilization, and protease fragments are compared between the solubilized or unsolubilized samples. Using a proteinase K protection assay of proteins translated in RRL supplemented with CMMs, Umigai et al<sup>19</sup> showed that the M2 domain of the  $K^+$  channel Kir 2.1 is oriented with the C-terminus toward the cytoplasm. A similar assay has been used to explore the effect of pathogenic mutations on the prion (PrP) protein.<sup>14</sup> In addition to determining topology of a particular protein, researchers can use protease assays to dissect the process of ER binding and translocation.<sup>20</sup>

### **Tagging Membrane Proteins to Determine Orientation**

Another strategy for determining membrane topology involves tagging a protein with an enzyme or epitope. Tagging is often used in conjunction with other studies such as glycosylation or protease assays to give a more complete picture of membrane topology. In one study, the C-terminal end of each of several deletion mutants of Presenilin I was tagged with *E. coli* leader peptidase (LPase). Anti-LPase antibody was able to immunoprecipitate in vitro translated protein from some but not all of the deletion mutants based on the location of the C-terminus of the protein (either cytosolic or luminal).<sup>21</sup> C-terminal and N-terminal glycosylation tags have also been used in experiments to investigate the topology of vitamin K epoxide reductase.<sup>22</sup>

## **Glycosylation**

### ***N-Linked Glycosylation of Membrane and Secreted Proteins***

Glycosylation studies in RRL supplemented with CMMs or SP cells can provide information about membrane topology. Portions of proteins translocated into the lumen of microsomes or SP cells are exposed to enzymes responsible for core N-linked glycosylation. N-linked glycosylation acceptor sites can be inserted into the protein, at the N- or C-terminus, for example. Any sugar residues that are added should be removed by the actions of glycosidases, such as endoglycosidase H, when microsomes containing the proteins are treated with detergents to allow the glycosidase access to the luminal portion of the protein. Such a strategy was used to determine the membrane topology of vitamin K epoxide reductase.<sup>22</sup> Zhang and Ling used sensitivity to peptide N-glycosidase (PNGaseF) to determine whether an 18 kDa protease-protected fragment from mouse P-glycoprotein is glycosylated.<sup>23</sup> Additionally, carrying out translation in RRL supplemented with microsomes in the presence or absence of tunicamycin (a glycosylation inhibitor) can allow comparison of glycosylated and unglycosylated forms of proteins produced in vitro.<sup>24</sup>

The membrane topology of polytopic proteins (proteins that span the membrane multiple times) can be especially difficult to predict. The Presenilin-1 protein is a polytopic protein predicted by hydrophobicity analysis to span the membrane from six to eight times. To determine membrane topology for Presenilin-1, a series of C-terminal deletions was made to remove predicted transmembrane regions of the protein. The truncated proteins were translated in vitro in the presence of microsomes. Endoglycosidase H sensitivity of protein from solubilized microsomes changed as deletions of the transmembrane domains altered the orientation of the protein in the membrane.<sup>21</sup> Combined with protease protection assays and epitope tag labeling at the C-terminus, these glycosylation results supported a seven-transmembrane domain structure with an additional membrane-embedded domain for Presenilin-1.

### ***O-linked Glycosylation of Cytosolic and Nuclear Proteins***

Secretory and membrane proteins are not the only proteins in the cell that are glycosylated. Many nuclear and cytosolic proteins are modified by *O*-linked glycosylation (*O*-GlcNAcylation).<sup>25</sup> RRL contains the enzymes and substrates necessary for the *O*-GlcNAcylation of proteins,<sup>26</sup> and addition of microsomes or SP cells provides the environment necessary for correct membrane-protein folding. To assess whether the insulin-responsive glucose transporter GLUT4 undergoes *O*-GlcNAcylation, GLUT4 cDNA was transcribed and translated in RRL supplemented with CMMs.<sup>25</sup> RRL was able to successfully modify GLUT4 protein.<sup>25</sup>

### **Lipid Modification and Acetylation of Proteins**

#### ***Glycosylphosphatidyl Inositol (GPI) Anchors***

Some proteins are anchored to the cell membrane by means of a glycosylphosphatidyl inositol (GPI) modification at the C-terminus.<sup>27</sup> Unlike proteins incorporated into the membrane by a transmembrane domain, proteins that are GPI-anchored are reversibly associated with the lipid bilayer. GPI anchoring does not appear to be necessary for cell survival, but it is necessary for development.<sup>28</sup> Proteins that are modified by the addition of a GPI anchor contain two signal sequences, one at the N-terminus that directs protein synthesis to the ER and a second at the C-terminus that directs the addition of the GPI-anchor by a transamidase activity.<sup>29</sup> Small nucleophilic compounds like hydrazine can substitute for GPI, providing a means to assess whether GPI modification has occurred by comparing the molecular weight of proteins translated in the presence or absence of hydrazine.<sup>30</sup> Human placental alkaline phosphatase (PLAP) is a GPI-anchored protein.<sup>24</sup> GPI-anchored mini-PLAP has been generated by numerous groups using nuclease-treated RRL supplemented with microsomal membranes from CHO, F9, EL4 or K562 cells,<sup>24,28,29,31-33</sup> demonstrating that GPI modification can be reconstituted in a cell-free system.

#### ***Prenylation***

Some proteins are modified by prenylation, the attachment of one or more isoprenoid groups, such as the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group, to a cysteine residue. Prenylation can mediate membrane association of some proteins, particularly the Ras-like GTPases, and protein-protein interactions (e.g., nuclear lamins).<sup>34</sup> Prenylated proteins can be produced and detected in RRL supplemented with the labeled isoprenoid precursor mevalonic acid after the translation reaction is complete; additionally proteins synthesized in RRL can be modified using photoactivatable analogs of isoprenoids.<sup>35-37</sup> Gel-based assays to detect changes in protein migration as a result of prenylation are also used, but these are indirect assays and are usually performed along with a labeling experiment. Most prenylation assays require autoradiography of the labeled lysate, which can take weeks or months. Benetka and colleagues have developed an *in vitro* prenylation assay using N-terminal GST-tagged proteins and detection of <sup>3</sup>H-labeled precursors using a TLC linear analyzer.<sup>38</sup> The incorporation of the GST tag allows the labeled protein of interest to be separated from free radioactive label and other proteins in the RRL, and using the TLC scanner to detect the incorporated lipid molecule significantly reduces the time required to obtain results.

#### ***N-Myristoylation and Palmitoylation***

Many proteins in eukaryotic cells are subject to N-myristoylation, the addition of a 14-carbon fatty acid to the N-terminus.<sup>39</sup> In addition to being prenylated, the alpha subunits of some G-proteins, including pp60<sup>src</sup> and p21<sup>ras</sup>, are myristoylated. Other G-protein alpha subunits, including some of the G<sub>s</sub> and G<sub>q</sub> subunits, are not myristoylated, but are instead modified by the attachment of a 16-carbon palmitic acid (palmitoylation), and others are both myristoylated and palmitoylated. Some studies suggest that N-myristoylation and palmitoylation contribute to the membrane association of these proteins.<sup>40-42</sup> N-myristoylation can occur cotranslationally, immediately after the removal of the N-terminal methionine from a protein or even post-translationally as with the protein BID (BCL-2 interacting domain), a substrate of caspase-8.<sup>39,43</sup> Upon cleavage

by caspase-8, BID reveals an N-myristoylation site. RRL contains the components necessary to complete N-myristoylation, and has even been used to myristoylate tumor necrosis factor, a normally nonmyristoylated protein, when it is modified to contain the N-myristoylation motif of other myristoylated proteins.<sup>44,45</sup> The *Arabidopsis* SOS3 protein involved in plant salt tolerance has also been synthesized and myristoylated in an RRL system.<sup>46</sup>

### ***N*-Acetylation**

A majority (70–85%) of the proteins found in the cytoplasm or nucleus of eukaryotes may be modified by N-acetylation.<sup>47,48</sup> Examples of N-acetylated proteins include ovalbumin, actin and cytochrome c. Acetylation is catalyzed by N-acetyltransferases cotranslationally after the initiator methionine has been cleaved.<sup>48</sup> Many proteins are also acetylated post-translationally at internal sites by acetyltransferase enzymes different from those involved in cotranslational modification.<sup>48</sup> Acetylation of modified tumor necrosis factor (TNF) has been demonstrated in RRL,<sup>47</sup> and acetylation in RRL can be inhibited by the use of S-acetyl-CoA, an analog of acetyl-CoA.<sup>49</sup>

### **Reconstituting ER-Associated Protein Degradation**

Conditions such as environmental stress, viral infection and the absence of required partner proteins can result in the accumulation of aberrantly folded proteins in the rough endoplasmic reticulum (RER). The RER has a “quality control” system that targets these misfolded proteins for degradation. This process, ER-Associated Degradation (ERAD), requires ATP and is distinct from the lysosomal degradation pathway in cells.<sup>50</sup>

RRL in conjunction with CMMs or SP cells has been used to reconstitute ERAD activity.<sup>15,51</sup> RRL has several advantages over intact-cell systems for such study. The protein of interest will be the only protein labeled in the RRL, making its degradation easy to follow.<sup>51</sup> Second, a variety of microsomal membranes can be used with RRL to reconstitute the activity.<sup>15,51</sup> Because hemin inhibits the proteasome activity of ERAD, degradation studies are best performed in RRL that does not contain exogenous hemin. Researchers have reported that RRL that works well for studies of degradation is usually poor for translation.<sup>51</sup> Additionally, since ERAD is ATP-dependent, the RRL will need to be supplemented with ATP and an ATP regeneration system,<sup>51</sup> and some authors report that excess unlabeled methionine seems to aid in reconstituting ERAD activity.<sup>52</sup>

RRL-based protein degradation systems have been used to investigate the synthesis, stability and degradation of a variety of wild type and mutant proteins. In one such study, tyrosinase ERAD was reconstituted in a commercially available RRL system supplemented with an ATP regeneration system.<sup>53</sup> Wild type and mutant tyrosinase associated with albinism were translated in the presence of SP melanocytes; the SP cells were isolated and then resuspended in RRL with the ATP regeneration system. Both proteins were degraded, although the mutant protein degraded at a higher rate than the wild type protein. RRL-based and rat cytosol-based degradation systems have also been used to investigate the degradation of Apoprotein B (apoB).<sup>54</sup> Aliquots of the transcription/translation reaction of HA-tagged apoB were incubated in the presence of an ATP regeneration system in fresh RRL or rat hepatocyte cytosol with or without proteasome inhibitors. Inhibition of apoB degradation was more obvious in the rat hepatocyte cytosol, presumably because RRL contains factors that interfere with the proteasome inhibitors. To assess the role of the chaperone protein, hsp90, in the degradation of apoB48, geldanamycin (GA), an antibiotic that competes for the ATP binding site on hsp90, was added to pelleted CMMs before the degradation assay. There was no significant decrease in the amount of apoB48 in the presence of GA, indicating that GA did inhibit degradation and hsp90 was required for apoB48 degradation.<sup>54</sup>

RRL has been used to reconstitute the degradation of  $\alpha_1$ -antitrypsin Z [ $(\alpha_1\text{AT})\text{Z}$ ].<sup>52</sup> Individuals who are homozygous recessive for a mutation resulting in a Glu<sup>342</sup> to Lys substitution have increased susceptibility to liver disease.<sup>52</sup> The amino acid substitution disrupts proper folding of  $(\alpha_1\text{AT})\text{Z}$ , and individuals susceptible to the liver disease are not able to degrade the misfolded protein efficiently. Mutant and wild type  $(\alpha_1\text{AT})\text{Z}$  degradation were examined using an RRL degradation assay system. The mutant  $(\alpha_1\text{AT})\text{Z}$  was degraded efficiently. Mutant protein produced in the presence

of salt-washed or puromycin-treated, salt-washed microsomes was also degraded, indicating that the full complement of RER proteins was not required for degradation.<sup>52</sup>

Several mechanisms have been suggested to control the targeting of proteins accumulated in the ER for degradation, including regulating the trimming of N-linked oligosaccharide chains. Oligosaccharide side chains can be modified by mannosidase I in the ER. Inhibiting this activity seems to stabilize misfolded proteins.<sup>15</sup> Wild type MHC class I heavy chain and a mutant heavy chain that lacks the N-linked glycosylation site but that can assemble into functional MHC class I molecules were translated in RRL in the presence of SP HT1080 cells.<sup>15</sup> The wild type protein was degraded more quickly than the mutant, indicating that glycosylation is important for ERAD.

## In Vitro Viral Assembly

The ability to reconstitute cotranslational assembly events and protein interactions using cell-free RRL systems can be extended to the study of in vitro mammalian viral protein assembly, viral protein interactions with other cellular components, and viral protein effects on translation. Early studies of viral protein assembly demonstrated that capsid proteins expressed in RRL were capable of self-assembling in vitro. For example, adenovirus type 2 fiber protein synthesized in RRL formed trimers without requiring additional viral proteins or components.<sup>55</sup> From this relatively well-defined, single-protein model, more complex viral protein interactions and assembly studies evolved. Human papillomavirus-like particles have been assembled in vitro from L1 capsid protein expression in RRL.<sup>56</sup> These particles also mimicked endogenous virus in its conformational epitope exposure, and antibodies generated against the in vitro-assembled L1 particles were effective in recognizing similar epitopes in patient samples. In addition to human papillomavirus, human hepatitis C (HCV) core viral capsid precursor structures were also generated de novo in reticulocyte lysates.<sup>57</sup> Cell-free systems have allowed detailed examination of HCV core capsid assembly processes and properties, whereas mammalian culture systems have been limited by low viral titers.

In vitro expression of Gag precursor proteins in RRL has allowed detailed examination of the more complex pathway for retroviral assembly, which includes not only protein interactions, but also plasma membrane interactions and budding. Viral capsid structures of immunodeficiency virus type 1 (HIV-1) have been assembled from the Gag precursor protein p55<sup>gag</sup> expressed in RRL, and these particles resembled immature HIV-1 viral structures.<sup>58</sup> Additional processing of proteins in viral capsids can include prenylation and glycosylation. Myristoylation of HIV-1 viral particles<sup>59</sup> and glycosylation of woodchuck hepatitis virus capsid proteins<sup>60</sup> have been investigated using cell-free RRL systems. These results illustrate the importance of cell-free protein expression in delineating processes involved in viral particle assembly pathways.

## Protein Microarray Technology

In the field of functional proteomics, protein microarrays are filling a niche for miniaturization coupled with high-throughput assay capability.<sup>61,62</sup> Protein microarray concepts are patterned after DNA microarrays, but immobilization of diverse types of proteins in a manner that preserves conformation and functionality is a complex and challenging problem to solve. Continuing advances in microarray surface chemistries coupled with improvements in protein production capabilities, sensitive detection methods, and instrumentation are accelerating the pace of development for protein microarrays. Microarray formats include printing proteins at high density on a glass slides or other solid surfaces, using miniaturized reaction chambers adapted for slides, and assaying samples in multiwell plates.

Currently two general types of protein microarray applications are being pursued: antibody- or peptide-based arrays and functional protein arrays. Antibody- or peptide-based arrays bind proteins of interest in given samples, such as serum, and can provide information about the amount and specificity for binding of such proteins. This is referred to as protein profiling. The search for disease biomarkers for diagnostic purposes and drug screening capabilities drives many of the innovations for these types of arrays. Another strategy is to use protein microarray formats

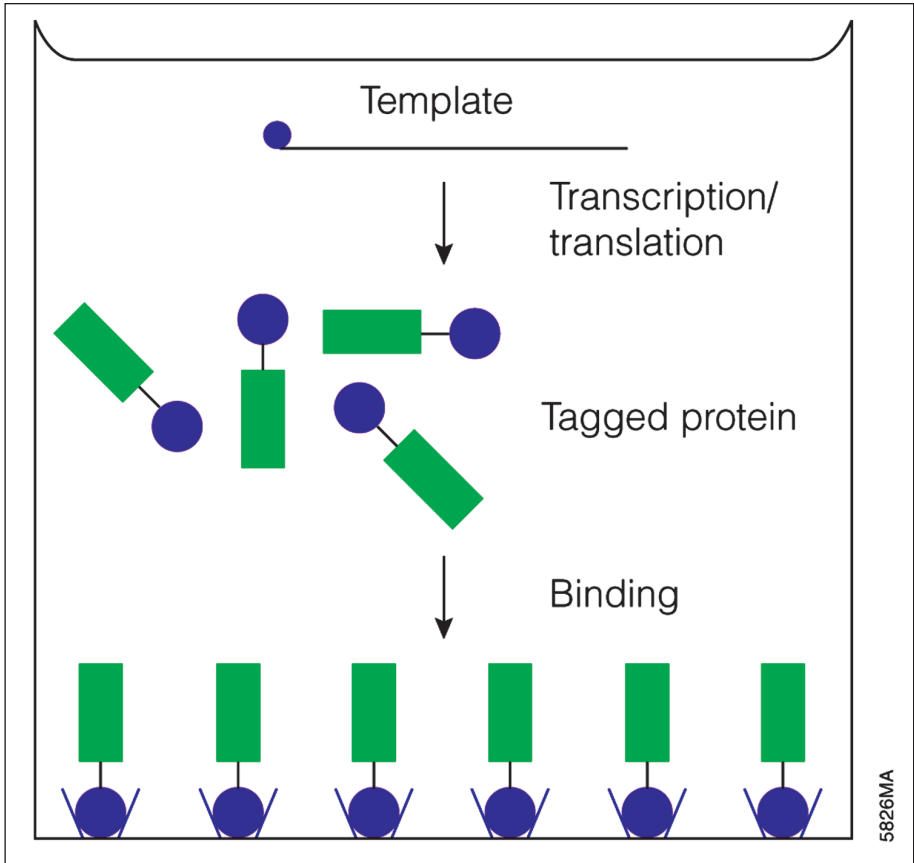


Figure 1. Schematic of multiwell format protein array experiment. Tagged proteins are generated through coupled translation and then bind to coated multiwell surface. Printed with permission from Promega Corporation.

to interrogate and carefully perturb protein functions such as protein-protein, protein-nucleic acid or protein-small molecule interactions, as well as protein enzyme activities. Cell-free protein expression systems, such as RRL, are well suited to supply the functional proteins required for these types of protein microarrays. Cell-free protein expression in RRL is versatile and allows incorporation of specific moieties into the protein sequence for immobilization or detection strategies, as well as post-translational modifications, in an automatable manner.

Early proof-of-principle for use of RRL in combination with a multiwell array-type format was demonstrated by He and Taussig in 2001,<sup>63,64</sup> and named "PISA" (Protein In Situ Array). Proteins with double (His)<sub>6</sub> tags were expressed directly from DNA templates with RRL in each well, and the expressed proteins were immobilized by the (His)<sub>6</sub> tag to nickel-coated surfaces. Expression and immobilization of functional proteins were demonstrated by enzymatic activity of a cloned (His)<sub>6</sub> tagged-luciferase and a tagged-single chain antibody fusion that bound its corresponding antigen, progesterone. Microwells offer an advantage of maintaining aqueous reaction conditions that are compatible with native protein conformation, compared to more harsh conditions present on a spotted glass slide. Expression of tagged protein expressed in cell-free extracts eliminates laborious protein purification schemes (Fig. 1).

Oleinikov et al<sup>65</sup> describe a variation on protein arrays based on expression of protein from RRL with concomitant immobilization. This strategy exploits features of electronic semiconductor microchips for protein binding and detection. A second variation of protein arrays takes advantage of the stability of immobilized DNA in microarrays, protein expression and spatially defined protein capture.<sup>66</sup> This approach, named NAPPA (nucleic acid programmable protein array) involves generating protein *in situ* with a coupled transcription/translation RRL layered onto slides printed with a mixture of biotinylated DNA, avidin and polyclonal GST antibody. Target proteins are expressed from template DNA encoding a C-terminal GST fusion tag and then the fusion proteins are spatially bound to the GST antibody. The target proteins are detected using a monoclonal antibody to GST.

## Protein Interaction with Other Molecules

Cell-free RRL has been used to examine numerous protein-protein,<sup>70</sup> protein-DNA,<sup>71,72</sup> and protein-RNA interactions<sup>73-75</sup> via immunoprecipitation<sup>67,74-76</sup> and tagged protein pull-down.<sup>68,70,72</sup> Confirmation of protein-protein interactions *in vitro* often includes expression of target proteins in RRL and pull-down with a tagged protein, such as GST-tagged protein, which is bound to a solid support. These types of GST-pull-down experiments have been used to confirm specific interactions of protein involved in signal transduction,<sup>77,78</sup> transcription regulation,<sup>79-81</sup> ion channels<sup>82</sup> and spliceosomes.<sup>83</sup> Reconstitution studies can be performed in which expressed proteins form a complex in cell-free RRL and give a measurable biochemical response that mimics an *in vivo* response. One interesting recent example is the demonstration that p43, a telomerase accessory protein, can affect the *in vitro* nucleotide addition activity and processivity of the conserved core consisting of the protein telomerase reverse transcriptase (TERT) and the telomerase RNA subunit. The resulting reconstituted ternary complex [TERT•RNA•p43] was identified and examined by immunoprecipitation in coupled transcription/translation in RRL.<sup>76</sup>

Another interesting example of protein-RNA interaction in RRL involved the depletion of the internal ribosome entry site (IRES)-interacting protein of the RRL by the immobilized foot-and-mouth virus (FMDV)-IRES.<sup>84</sup> The effect of the depleted lysate was assessed by translation efficiency of transcripts that were either capped or had FMDV IRES in the sense or antisense orientation. This procedure should be useful for analysis of protein-RNA interactions and their role in IRES-dependent translation.

Cell-free translations in RRL can also provide information about protein-protein interactions. Translation in RRL with microsomal membranes and immunoprecipitations have helped to elucidate the mechanism of activation of the endogenous p21-activated kinase 2 (PAK-2) by Nef proteins that are encoded by human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) *in vitro*.<sup>67</sup> The cell-free system allows further investigation of the molecular mechanism of activation because the timing and order of component additions can be easily controlled.

Protein interactions that involve ribosome-associated chaperones (cotranslational folding and targeting) and post-translational interactions have also been explored using RRL.<sup>68-70</sup> During cotranslational folding the nascent chains interact with chaperones such as the 70-kD heat shock protein cognate (Hsc-70) and nascent polypeptide-associated complex (NAC)<sup>68,85</sup> and chaperonins such as the Tailless complex polypeptide 1 (TCP1) ring complex (TRiC).<sup>61,87</sup> Recently the interaction of the TRiC with the nascent polypeptide chain as it emerges from the ribosome was demonstrated using photoreactive N<sup>ε</sup>-(5-azido-2-nitrobenzoyl)-Lys-tRNA<sup>lys</sup> along with translation of truncated actin in the RRL. Post-translation interactions of chaperones have been investigated using mutagenesis and immunoprecipitation from RRL after the expression of protein kinases. These studies showed that phosphorylation of Ser<sup>12</sup> of the Hsp-90 cochaperone Cdc37 is critical for its interaction with eukaryotic protein kinases and Hsp-90.<sup>70</sup>

Historically, protein-DNA interactions have been identified via mobility shift assays<sup>71,72</sup> in which the DNA binding activity of proteins expressed in RRL are visualized by a shift of molecular weight on native polyacrylamide gels. Human biliverdin reductase (hBVR) is a serine/threonine kinase that catalyzes the reduction of biliverdin to bilirubin in response to oxidative stress. Using



hBVR and hBVR mutants that were translated in RRL and analyzed using a mobility shift assay, hBVR was found to bind to specific DNA sequences.<sup>71</sup>

## Display Technologies

Cell-free display technologies, such as ribosome display,<sup>86-91</sup> mRNA display<sup>92-94</sup> or in vitro virus (IVV),<sup>95</sup> and in vitro compartmentalization (IVC)<sup>96</sup> are powerful technologies that can be used to identify protein-target molecule interactions and for directed evolution of proteins for desired improvements. These technologies rely on coupled transcription/translation or translation using RRL or other sources of lysates. Cell-free display technologies have advantages over cell-based display technologies such as phage display,<sup>97</sup> and cell surface display on bacteria<sup>98</sup> or yeast.<sup>99</sup> The cell-based display systems have limited library diversity because of transfection inefficiencies, the inability to specify incorporation of nonnatural amino acids via amber suppressor tRNAs and bias against cytotoxic proteins. Display technologies rely on coupling genotype (mRNA) to phenotype (protein) to retrieve the genetic information along with protein function.

### *Eukaryotic Ribosome Display*

Eukaryotic ribosome display is an entirely cell-free technology that screens and selects functional proteins and peptides from large libraries. For ribosome display, the link between genotype and phenotype is accomplished by an mRNA-ribosome-protein (PRM) complex that is stable under controlled conditions. The eukaryotic method of ribosome display using RRL for coupled transcription/translation has been used to display single-chain antibodies to form an antibody-ribosome-mRNA (ARM) complex.<sup>87,88,91</sup> The function of the single-chain antibody is evaluated by its binding properties to an immobilized antigen. The function of other non-antibody proteins can be evaluated by using a different immobilized target, such as a partner protein, ligand or substrate, to capture the relevant PRM complexes. The mRNA that is complexed with the protein can then be amplified by reverse transcription polymerase chain reaction (RT-PCR) and recovered as DNA. If screening and selection is the goal, then proofreading DNA polymerases are necessary; however, if evolution or diversification of the DNA sequence pool is required, then a nonproofreading polymerase such as *Taq* DNA polymerase is used. The major distinguishing feature between eukaryotic ribosome display and prokaryotic ribosome display<sup>86,90</sup> is that in eukaryotic ribosome display, the RT-PCR is carried out on the intact PRM complexes rather than on mRNA that has been released from PRM complex.

Eukaryotic ribosome display has been used to select the enzyme, sialyltransferase II, from a cDNA library in a 96-well plate coated with the substrate, ganglioside GM3.<sup>100</sup> Coupled transcription/translation in an RRL expression system from the cDNA library resulted in an enzyme-specific protein-ribosome-mRNA (PRIME) complex. A recently described modification of eukaryotic ribosome display incorporates Q $\beta$  RNA-dependent RNA polymerase into the display and selection process.<sup>101</sup> This allows a continuous in vitro evolution (Fig. 2). The cell-free RRL is used in the coupled transcription/translation mode to generate mRNA and then protein. The Q $\beta$  RNA-dependent RNA polymerase mutates the generated mRNA and thus the simultaneous display of the protein generated from the original mRNA. The ribosome ternary complexes display the synthesized proteins/single chain antibodies and are selected against immobilized antigens. For the selection process, the displayed wild type and mutants are competing for the target. The recovery of the mRNA is the same as in ARM display.

Recently, improvements have been developed for eukaryotic ribosome display that allow 20.8-fold more efficient selection<sup>102</sup> than current methods, making ribosome display a readily accessible technique for all researchers.

### *mRNA Display or in Vitro Virus*

A different approach to the selection and identification of functional proteins is mRNA display, also called in vitro virus,<sup>95</sup> a technique that uses the cell-free RRL translation system to link a peptide or protein covalently to its encoding mRNA.<sup>92-94</sup> The mRNA has a puromycin-tagged DNA linker ligated or photo-crosslinked to the 3'-terminus.<sup>103</sup> The ribosome will stall when

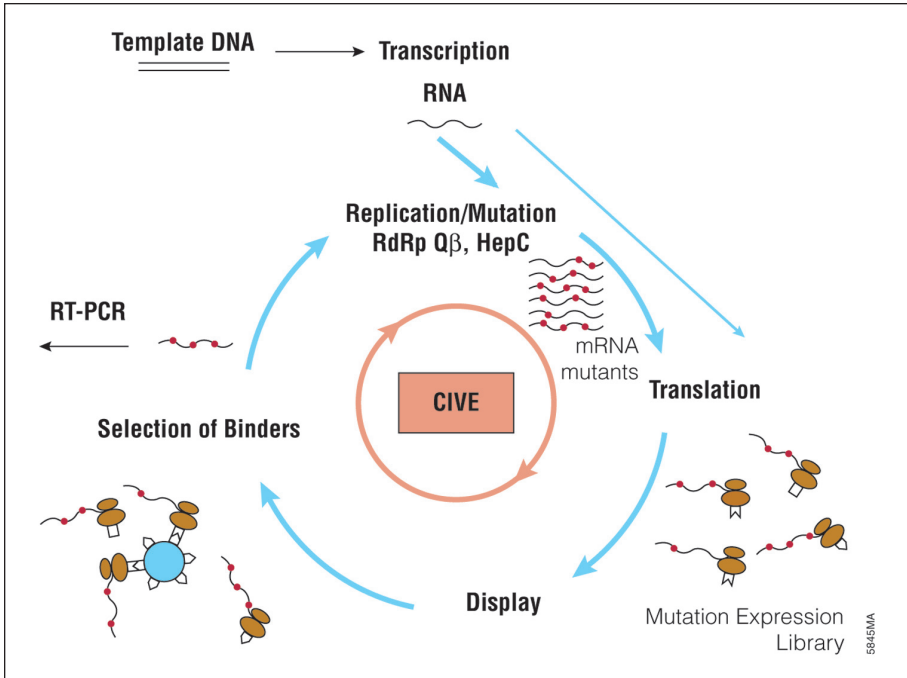


Figure 2. The proposed continuous in vitro evolution (CIVE) cycle. The coupled transcription/translation system with inclusion of Q $\beta$  replicase. This produces a process in which, in the reaction mix, mRNAs are transcribed from the DNA template by T7 polymerase, replicated and mutated by the Q $\beta$  replicase, translated and displayed on the surface of the ribosomes. In selecting against a target, the displayed wild type and mutant are competing for the target. Reprinted from: Irving RA et al, *J Immunol Meth* 248:31-45; ©2001 with permission from Elsevier.<sup>101</sup>

it reaches the mRNA-DNA junction, and puromycin enters the ribosomal A-site. The nascent peptide is coupled to the puromycin by the peptidyl-transferase. The complex of peptide-puromycin-DNA linker-mRNA is dissociated from the ribosome and can then be used for selection and identification of functional protein (Fig. 3). mRNA display or IVV in combination with in vitro selection are powerful tools for evolving and discovering new functional proteins. mRNA display of a random peptide library has been used to determine the epitope-like consensus motifs that define the determinants for binding of the anti-c-Myc antibody.<sup>104</sup> Recently, a method has been described for mRNA display using a unidirectional nested deletion library.<sup>105</sup> The method identified high-affinity, epitope-like peptides for an anti-polyhistidine monoclonal antibody and should be useful for determining minimal binding domains and novel protein-protein interactions. Also, mRNA display can be used to select mRNA templates capable of efficiently incorporating the nonnatural amino acid, biotinyl-lysine, a lysine derivatized at the epsilon amino via amide linkage to biotin.<sup>106</sup> To generate the mRNA-peptide fusions, the template library and the tRNA pools are added to RRL for translation. The mRNA-peptide fusions contain a mixture of peptides, some of which contain the biotinyl-lysine. Those that contain biotinyl-lysine can be purified by binding to streptavidin-agarose.

### ***In Vitro Compartmentalization***

In vitro compartmentalization (IVC) links genotype and phenotype by compartmentalization into discrete water-in-oil emulsions.<sup>96</sup> Until recently, IVC has mainly been used in conjunction

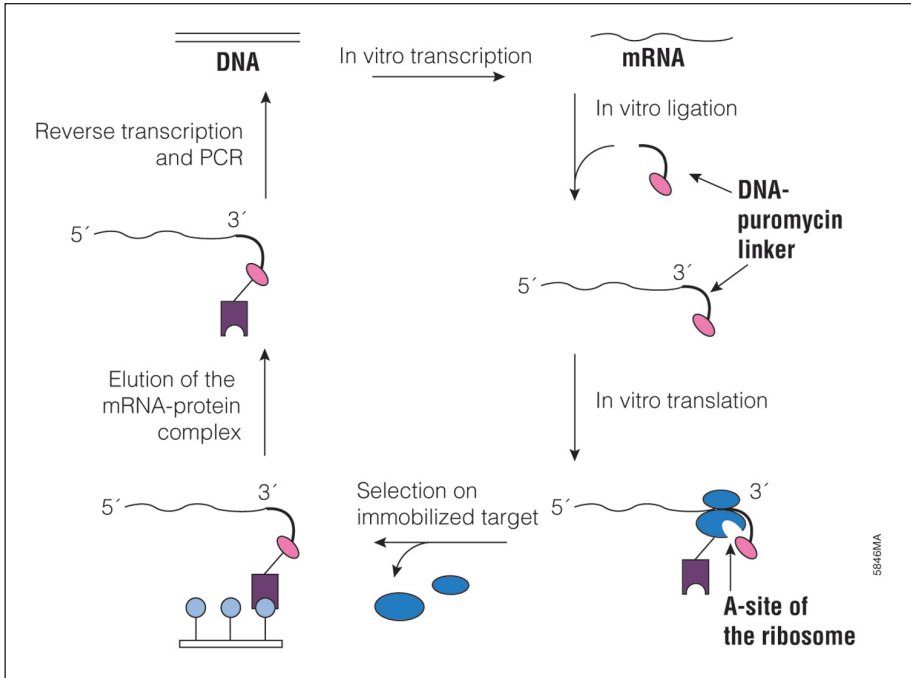


Figure 3. Protein:RNA fusion. Covalent RNA:protein complexes can be generated by ligation of a DNA:puromycin linker to the in vitro transcribed mRNA. The ribosome stalls at the RNA:DNA junction. Puromycin then binds to the ribosomal A site. The nascent polypeptide is thereby transferred to puromycin. The resulting covalently linked complex can be used for selection experiments. Reprinted from: Schaffitzel C et al, J Immunol Meth 231:119-135; ©1999 with permission from Elsevier.<sup>90</sup>

with prokaryotic coupled transcription and translation (*E. coli* S30 extract) for selection of peptide ligands<sup>107-109</sup> and directed evolution of *Taq* DNA polymerase,<sup>110</sup> bacterial phosphotriesterase<sup>111</sup> and DNA methyltransferase.<sup>112</sup> However, IVC has been used in conjunction with coupled transcription/translation in RRL to select active restriction enzymes from a randomized large ( $10^9$ – $10^{10}$  molecules) mutant *Fok* I library.<sup>103</sup> Use of RRL in this way is made possible by a new inert emulsion formulation that is compatible with coupled transcription/translation,<sup>114</sup> allowing an expanded range of vertebrate protein targets that may be difficult to express as soluble and functional in S30 (bacterial) or wheat germ lysates.

## Screening

### *In Vitro* Expression Cloning (IVEC)

IVEC<sup>115,116</sup> is another approach that uses RRL for coupled transcription/translation to identify genes and elucidate protein interactions with other molecules. Using this method cDNAs or small plasmid pools (50–100 clones) are expressed and then assayed for a specific function. Plasmids from the positive pools are further deconvoluted and rescreened (Fig. 4). The process is repeated until a single positive plasmid has been identified. IVEC is only successful if the specific function assayed can be distinguished from the endogenous activity in cell-free RRL. IVEC has been successfully used to identify protein substrates,<sup>117-120</sup> protein-protein interactions,<sup>121,122</sup> enzymatic activity,<sup>123-125</sup> protein-DNA interactions,<sup>126</sup> and phospholipid-protein interactions.<sup>136</sup>

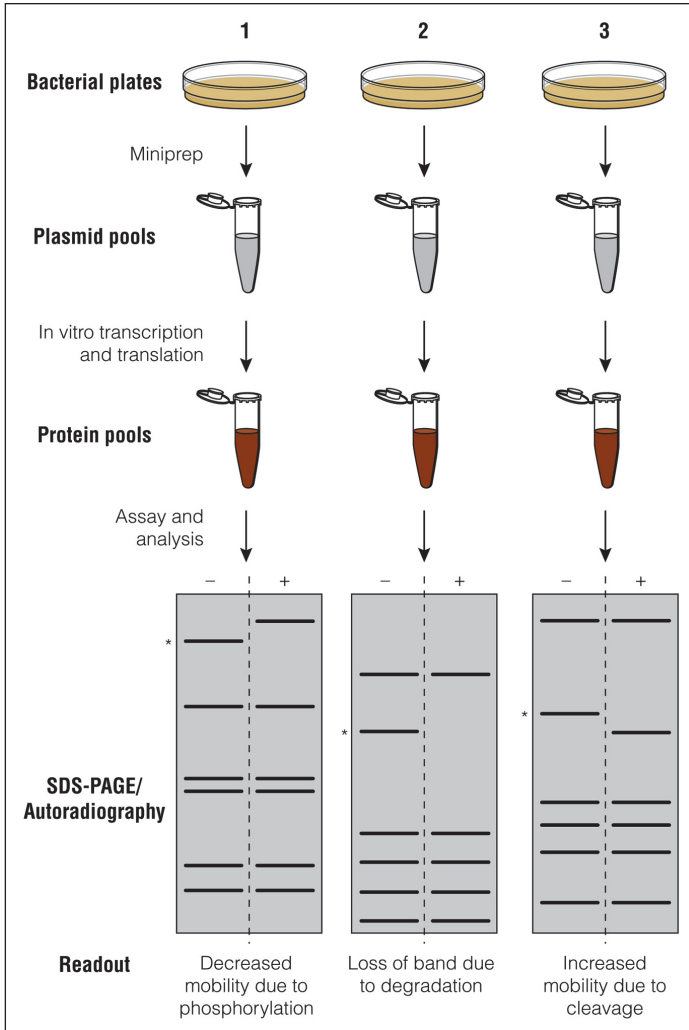


Figure 4. The strategy of in vitro expression cloning. An unamplified cDNA expression library is plated at a density of approximately 100 clones per bacterial plate. Pooled plasmid DNA is obtained by scraping colonies from each plate and performing a small-scale plasmid purification. Each plasmid pool is then transcribed and translated in vitro with a commercially available system, such as the TnT<sup>®</sup> Coupled Transcription/Translation Systems from Promega Corporation. The resulting protein pool is then assayed for the presence of an activity. In the illustrated experiment, a radioactive amino acid is included in the translation system to specifically label the pool of proteins. Incubation of a pool with a modifying enzyme (lanes labeled +) such as a protease or kinase can result in a change in mobility of a substrate (bands marked with asterisk). Pool 1 contains a protein whose mobility is reduced following treatment with a kinase; Pool 2 contains a protein that is degraded following treatment with an extract containing an activated proteolytic system; Pool 3 contains a protein that is specifically cleaved following treatment with a protease, decreasing its apparent molecular mass. Once a pool containing a candidate activity is identified, the original cDNA pool is subdivided and retested until the single cDNA encoding the protein of interest is isolated. Reprinted with permission from: King RW et al, *Science* 277:973; ©1997 AAAS.<sup>115</sup>

### Protein Truncation Test

The protein truncation test (PTT) is a mutation detection technique that specifically identifies pathogenic premature termination codons and has the advantage of not detecting polymorphisms. Using extracted RNA, the coding region is screened for truncated mutations. The RNA is subjected to RT-PCR such that the cDNA product contains a T7 promoter. The cDNA is subjected to coupled transcription/translation in cell-free RRL, and the translated products are analyzed on gels to identify the truncated proteins. The PTT has been applied to screening for many clinical conditions including hereditary breast and ovarian cancer (BRCA I and BRCA II),<sup>128</sup> colorectal cancer (APC),<sup>129</sup> Duchenne Muscular Dystrophy (DMD),<sup>130</sup> and neurofibromatosis type I (NFI).<sup>131</sup>

### Other Screening

An approach to screening that uses cell-free RRL and site-directed mutagenesis to identify elements critical for protein N-myristoylation<sup>132</sup> has recently been described. Sequential vertical-scanning mutagenesis in the N-terminal region of tumor necrosis factor (TNF), followed by cotranslation N-myristoylation in RRL revealed the major sequence requirements for protein N-myristoylation. RRL has also been used to functionally screen a randomly mutagenized phage library.<sup>133</sup> In this example, critical amino acids in the protein C1 were identified that were responsible for binding RNA. Other amino acids were identified that were important for protein oligomerization. Protein C1 is a member of the heterogeneous ribonucleoproteins that bind nascent RNA transcripts.

Screens that use cell-free RRL have also been developed to identify small-molecule inhibitors of translation.<sup>134</sup> Numerous reports have implicated alternative translation initiation controls occurring in cancer cells,<sup>135-139</sup> and small-molecule inhibitors may provide tools for determining the molecular mechanism of this alternative regulation. A high-throughput screen was designed to identify small-molecule inhibitors of eukaryotic translation as well as inhibitors that interact at the mRNA-ribosome level to inhibit gene-specific translation. This multiplex in vitro translation was used to screen over 900,000 distinct compounds identified novel inhibitors.<sup>134</sup> A secondary high-throughput eukaryotic translation screen to discover broad spectrum antibacterial compounds has also been used to assess the biochemical selectivity of the compounds for prokaryotic translation.<sup>140</sup>

Another screen using RRL is a PCR-based rapid detection screen for pyrazinamide (PZA)-resistant *Mycobacterium tuberculosis*.<sup>141</sup> After amplification of the *pncA* gene and coupled transcription/translation of the PCR product, the activity of the enzyme pyrazinamidase is measured by the conversion of PZA to pyrazinoic acid. Other PCR-based coupled transcription/translation methods for rapid phenotypic screening have also been developed, including screening of the thymidine kinase gene for monitoring acyclovir resistant herpes simplex virus and varicella-zoster virus.<sup>142</sup>

### Conclusions

Cell-free RRL plays an important role in functional proteomics, whether the protein is destined for the ER, modification, degradation, or forms a complex with DNA, RNA and other proteins. RRL provides a mammalian environment for elucidating quasi-cellular mechanisms and can be easily manipulated by depleting or adding protein, tRNA or membranes to provide the desired environment so that the function of a protein or proteins can be studied. Additionally, cell-free RRL is proving to be a useful tool for high-throughput protein synthesis in protein microarrays and other screening situations.

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