Recent advances in producing and selecting functional proteins by using cell-free translation

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Prokaryotic and eukaryotic *in vitro* translation systems have recently become the focus of increasing interest for tackling fundamental problems in biochemistry. Cell-free systems can now be used to study the *in vitro* assembly of membrane proteins and viral particles, rapidly produce and analyze protein mutants, and enlarge the genetic code by incorporating unnatural amino acids. Using *in vitro* translation systems, display techniques of great potential have been developed for protein selection and evolution. Furthermore, progress has been made to efficiently produce proteins in batch or continuous cell-free translation systems and to elucidate the molecular causes of low yield and find possible solutions for this problem.

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Abbreviations

AURE	AU-rich element
CFCF	continuous-flow cell-free
PEP	phosphoenolpyruvate
PABP	poly(A)-binding protein
SFCF	semicontinuous-flow cell-free
UTR	untranslated region

Introduction

Over several decades protein translation *in vitro* has been used mainly to decipher its mechanism — perhaps the most amazing process in a living cell, the coupling of information stored in polynucleotides to the manufacture of functional proteins. More recently, a renewed interest in applying *in vitro* translation to biotechnology has been seen. The range of new applications based on cell-free translation encompasses the rapid screening of many mutants by the direct translation of PCR products, the introduction of unnatural amino acids, and the study of complex protein assembly processes, including those of membrane proteins. These applications rely on the correct folding of the *in vitro* expressed polypeptide into its three-dimensional structure. A number of studies over the past few years have investigated some of the factors which allow for correct protein folding in a cell-free system, including molecular chaperones and disulfide-forming catalysts.

Directly related to the possibility of producing correctly folded proteins *in vitro* is the exciting prospect of carrying out molecular selection and evolution *in vitro*. A number of recent studies have shown that the translated and folded polypeptide can be made to remain on the ribosome, which in turn remains complexed with the corresponding mRNA, and the protein can be selected from a diverse library for ligand-binding properties. Through multiple rounds of *in vitro* mutagenesis and *in vitro* selection, proteins with improved properties can be obtained.

While theses studies exploit very powerful affinity separation on a small scale, great progress has also been made at the other end of the spectrum with cell-free translation on large scales, notably using flow reactors. The more difficult it is to make a desired protein *in vivo* (e.g. membrane proteins or complex assemblies), or even impossible (e.g. variants with unnatural amino acids or toxic proteins), the more attractive is the possibility of using cell-free translation for this purpose.

Whereas a number of articles have summarized important aspects of this field [1,2], we are not aware of recent reviews covering the whole area. To put the current work on a firm foundation and in proper perspective, therefore, we cite a higher proportion of older articles than may be customary in this series. We will first review the state of the art and recent progress in optimizing the efficiency of cell-free translation in both prokaryotic and eukaryotic systems. Particularly, some of the factors limiting translation efficiency have been pinpointed, even though cures for all problems have not been found. Next we will summarize the present technology of continuous flow reactor systems. The question of how proteins can efficiently reach the native state *in vitro*, obviously central to the whole field, will then be discussed, followed by a range of applications from complex membrane assemblies to the incorporation of unnatural amino acids. The final part of the review deals with *in vitro* selection of peptides and proteins which have been made to remain associated with the ribosome and/or their mRNA, thereby coupling phenotype and genotype *in vitro*.

Efficient *in vitro* translation systems

Cell-free protein systems in batch configuration

General considerations

We will focus primarily on the typical translation systems in use today and discuss some of the factors influencing the amount of total protein made. The most efficient cell-free protein synthesis systems are derived from *Escherichia coli*, rabbit reticulocytes or wheat germ (reviewed in [1,2]). They are all based on crude cell lysates, as the individual preparation of all necessary components (initiation, elongation and release factors, aminoacyl-tRNA-synthetases, tRNAs, enzymes for energy

regeneration, etc.) would be prohibitive. Normally, these systems are used as single batch reaction mixtures with a constant volume.

A typical *in vitro* system isolated from *E. coli* cells consists of a crude cell extract, the so-called S30 fraction (indicating the soluble fraction when centrifuged at 30,000g) which contains all the enzymes and factors necessary for transcription and translation, and mRNA [3]. Pre-incubation of the crude cell extract ('run off' process) is sufficient to remove endogenous messengers from the ribosomes, which presumably become destroyed by ribonucleases in this preincubation, in order to limit the newly synthesized proteins to molecules derived from the added nucleic acid. The *E. coli* system functions well in a temperature range of 24–38°C with the optimum at 37°C. Wheat germ extract can be directly used for expression of exogenous templates because of the low level of endogenously expressed messengers [4]. Here, the optimum temperature is in the range of 20–27°C, but can be increased to up to 32°C for higher expression of some templates [5]. Reticulocyte lysate is prepared by directly lysing blood cells of anemic rabbits, thus increasing the number of proerythrocytes or reticulocytes which are subsequently treated with micrococcal, Ca^{2+}-dependent RNase to remove endogenous mRNA [6]. This system works in a temperature range of 30–38°C.

It should be noted that the reaction temperature has not only an effect on the enzymatic process of translation and mRNA degradation, but also on the folding of the synthesized proteins. Thus, statements about the general performance of the different systems must address these issues, and the answer will almost certainly be protein-dependent. Usually, lower temperatures will lead to higher yields in the absence of chaperones, but not necessarily in their presence. The overall protein production in these *in vitro* systems is of course template-dependent and is usually in the range of 0.1–20 μg ml^{-1} reaction mixture. All extracts may be obtained from a number of commercial sources, however, at quite significant costs.

Protein synthesis *in vitro* will continue until one of the components is either depleted, degraded, or becomes inhibiting. The most serious problems leading to termination of protein synthesis are, firstly, a fast depletion of energy sources, resulting in starvation of nucleotide triphosphates [7•,8] and the consequent inhibition of protein synthesis by small molecule by-products, such as the hydrolysis products of triphosphates [9–11], secondly, mRNA degradation (for example, see [12]) and finally, in some cases, inhibition by the synthesized polypeptide itself. The inhibiting effect of free Ca^{2+} ions released during the translation reaction, possibly from decaying polyphosphate compounds, was also shown [13]. Ca2+ ions which might trigger the phosphorylation and subsequent inactivation of the α-subunit of initiation factor 2 (eIF-2) [14].

Energy supply as a limiting factor

The biochemical energy in a cell-free protein synthesis system is supplied by the hydrolysis of triphosphates. For efficient translation, the triphosphate concentration is maintained by an energy regeneration system. For ATP and GTP regeneration, creatine phosphate with creatine phophokinase are usually used in eukaryotic cell-free systems, whereas for bacterial cell-free systems the combination of phosphoenolpyruvate (PEP) with pyruvate kinase has been used traditionally. Another regeneration system based on *E. coli* acetate kinase and acetyl phosphate [15], however, may have the advantage that the ATP level is maintained twice as long as in the case of PEP and pyruvate kinase. Because acetate kinase is present at sufficient levels in bacterial extracts, it does not need to be added exogenously in the *E. coli* system.

Studies of the biochemical energy levels in different cell-free systems have detected a high rate of triphosphate hydrolysis, to mono- and diphosphates, during protein synthesis in wheat germ extract, even in the presence of energy regeneration systems [7•], as well as in an *E. coli* S30 extract [12]. The hydrolysis of more than 80% of the ATP and GTP initially present occurs independently of protein synthesis, as measured in the wheat germ system. It was suggested that acid phosphatases are responsible for the nonspecific hydrolysis of the nucleotide triphosphates [7•,16]. The authors proposed to improve the biochemical energy efficiency by removing or inactivating these acid phosphatases, but this has not yet been done in practice.

Once translation has stopped in a wheat germ system, protein synthesis activity can be recovered by removing low molecular weight (MW) components, such as the hydrolysis products of nucleotide triphosphates, and by replenishing the energy [7•]. Another approach to increase translation yield in a wheat germ cell-free system has used a preincubation without RNA to trigger the formation of the preinitiation complex, formed from initiation factor eIF-2, GTP, aminoacyl-tRNA and the ribosomal 40S subunit (which is the rate-limiting step in the initiation of protein synthesis). Subsequent addition of mRNA and replenishment of energy and amino acids leads to higher yields than by directly starting mRNA translation [17].

Recent extensive studies have focused on the composition of the reaction mixture and methods to prepare the cell extracts in order to prolong protein synthesis and, as a consequence, increase protein yield [18–20]. It was found that the productivity of a batch-mode cell-free protein synthesis system could be significantly improved by simply increasing the concentration of the components in the reaction mixture, such as ribosomes, PEP, and polyethylene glycol. Condensed extracts of both *E. coli* and wheat germ have been prepared by concentrating the original preparation with ultrafiltration membranes with different molecular cut-offs [19], with a cut-off of 300 kDa showing the highest productivity (~30 μg ml^{-1} h^{-1} production of

dihydrofolate reductase protein) and the lowest specific activity of acid phosphatases, which may have been dialyzed out. As the crude ribosome preparation pelleted from the *E. coli* S30 extract by ultracentrifugation is sufficient for efficient *in vitro* synthesis of proteins, the factors required for translation must be generally associated with ribosomes [21]. The highest level of production per volume in the batch system was demonstrated when the *E. coli* extract was condensed eightfold with an ultrafiltration membrane with a molecular cut-off of 10 kDa and combined with an increased concentration of PEP and polyethylene glycol. The level of newly synthesized active chloramphenicol acetyltransferase reached was 0.3 mg ml^{-1} [20]; however, there was only a marginal net increase of produced protein per amount of cell extract.

mRNA production, translation and degradation

Protein production *in vitro*, especially on a preparative scale, requires relatively large amounts of mRNA. The eukaryotic mRNA 5′-cap structure ^{7m}GpppN, where N can be any nucleotide) has an important role for stability and translation, and capping of mRNA improves translational efficiency. The same level of produced protein, however, can be reached by using a 2–3 times higher concentration of uncapped and, therefore, considerably cheaper mRNA. Preparative amounts of both capped and uncapped mRNA can be produced with phage RNA polymerases from either circular or linearized plasmids or from PCR products (for a review and protocols see [22,23••,24]).

mRNA can also be made directly in the translation reaction in a so-called coupled transcription/translation reaction with either endogenous *E. coli* RNA polymerase or exogeneous phage RNA polymerases in bacterial systems [25–27] or exogeneous phage RNA polymerases in eukaryotic systems [28,29], obviously requiring a DNA template with the appropriate promoter. In bacterial extracts, the endogenous *E. coli* RNA polymerases can be more efficient in coupling transcription and translation than the highly productive phage RNA polymerases, which can overload the translational machinery of *E. coli* and lead to mRNA not protected against nucleases by the ribosomes. In the latter case, mRNA accumulates faster than it gets processed in translation and the higher amount of mRNA does not result in more protein product. Secondary and tertiary structures of some mRNAs can decrease the ability of the ribosomes to bind to the translation initiation site, whereas in a tight coupling between transcription and translation ribosomes are able to initiate translation as soon as nascent mRNA protrudes out of the RNA polymerase [30]. The ratio of mRNA to the amount of ribosomes, therefore, needs to be optimized by varying the amount of phage polymerase added exogenously or made from its corresponding DNA [12,27], as an excess of mRNA will inhibit translation, especially in eukaryotic systems. mRNA templates can also be transcribed from linear or circular plasmids or PCR products, even in such coupled transcription/translation systems [26,31•,32,33]. Transcription is generally carried out under reducing conditions, as RNA polymerases are inactivated by oxidation. Thus, if the optimal folding of the protein requires oxidative conditions, separate reactions must be used. Another attractive possibility to produce high amounts of mRNA directly in the translation mixture is the use of co-translational mRNA replication with Qβ RNA polymerase which uses RNA as a template (reviewed in [34]).

The translational efficiency of mRNA depends on its structural features. Most cDNA sequences can be sufficiently well expressed without any addition of translational enhancers. The sequence of interest needs only to be provided with a favorable Kozak sequence in eukaryotic and the Shine-Dalgarno sequence in prokaryotic cell-free systems, the respective sequences upstream of the ATG codon that are responsible for translation initiation. The most preferred AUG context for plant and animal genes is AACAAUGGC. The critical positions in both cases are a purine at position -3 and a guanine at position +4 (where A at the AUG is +1) [35,36]. In prokaryotes, the interaction of the Shine-Dalgarno sequence upstream of the start codon with the 16S rRNA of the small ribosomal subunit directs ribosomes to the initiation site and is, therefore, essential for efficient translation in the *E. coli* system (reviewed in [37]).

The stability of mRNA is one of the major parameters limiting the efficiency of protein synthesis in a cell-free system where endogenous ribonucleases are present. The degradation of template RNA becomes a serious problem in the *E. coli* cell-free translation system, even if partially ribonuclease-depleted extracts (e.g. *E. coli* strain MRE600, lacking RNase I) or RNase inhibitors are used. The rapid degradation of mRNA favors the use of coupled S30 *E. coli* transcription/translation systems where a high steady state level of mRNA can be preserved by ongoing transcription. It is, therefore, the method of choice for obtaining maximal yields unless the redox requirements for transcription and the folding of the synthesized protein are different, such as for proteins with disulfide bonds. mRNA could, however, also be stabilized by chemical modifications using nucleotides with 2′-O-acetyl groups (as shown with a wheat germ cell-free system [38]) and with a phosphorothioate backbone (in *T. thermophilus* and *E. coli* cell-free systems [39,40]). The phosphorothioate-containing mRNA, where only one NTP was substituted with the corresponding NTPαS, functioned as a stable template in a prokaryotic cell-free translation system, which apparently resulted from the increased mRNA stability against nucleolytic attack, and the translation system produced functional products identical to those obtained with unsubstituted mRNA [39]. The single substitution of adenosine residues resulted in the highest stability and translation activity of thio-modified mRNA [39]. Yet, the stability of thio-modified RNAs seems to be sequence-dependent and, as a consequence, the half-life of differently substituted mRNA constructs in the *E. coli* cell-free system varies from 5 to more than 15 min [39,40].

In bacterial extracts, mRNA can also be stabilized by the introduction of secondary structural elements at the 5′ and 3′ ends. The 3′ stem-loop structures, such as repetitive extragenic palindromic (REP) sequences and rho-independent transcription terminators, can protect RNA from 3′-exonucleolytic digestion by 3′-5′-exonucleases such as PNPase or RNase II, two major exonucleases involved in mRNA turnover (for review see [41]). Also, the 5′-leader sequence of stable transcripts, such as that from phage T4 gene 32 or *E. coli omp*A, might stabilize mRNA. Two elements of the *omp*A transcript, the 5′-terminal stem-loop and the single-stranded RNA segment (ss2) containing a ribosome-binding site highly complementary to 16S rRNA were found to be responsible for this stabilizing effect. The bound ribosomes are believed to hinder attack of RNase E [42].

In wheat germ or reticulocyte lysates, however, there appears to be little *in vitro* degradation of mRNA during cell-free translation, and the degradation pathway is slowed down *in vitro*, compared to the *in vivo* situation. *Cis*-acting elements on the mRNA can influence its stability through the interaction with *trans*-acting protein factors, other than RNases themselves. At least three *cis*-elements can affect stability of many mRNAs, namely the poly(A) tail, AU-rich elements (AUREs) and oligo(U) regions in some 3′-untranslated regions (UTRs) (reviewed in [43]). Many AUREs can function in concert with AURE-binding proteins as destabilizing elements *in vivo* and *in vitro*. As an example, AUF1, a member of the AURE-binding protein family, binds an AURE as a hexameric protein [44] and serves as a signal for an mRNA-degradation pathway. The poly(A) sequence, attached to the 3′-end of most eukaryotic mRNAs, also influences RNA expression as well as RNA stability in the cytoplasm and can protect mRNA from rapid degradation by complex formation with a poly(A)-binding protein (PABP) (for review see [43]). The deadenylation of mRNA can trigger mRNA decapping, and mRNAs without caps are more unstable in cell-free systems. It was found that the complex between PABP and the poly(A) tail can physically interact with the capped 5′-UTR of the same mRNA, and this interaction may decrease RNA turnover as well as increase translation [45]. In yeast [46••], PABP interaction with the 5′-UTR is mediated by the elongation factor eIF4G, which is part of the cap-binding initiation factor eIF4F. In plants, eIFiso4G, the isoform of elongation factor eIF4G, appeared to be involved in the interaction with PABP [47••]. In mammalian cells, the interaction between PABP and the 5′-UTR might be mediated by the new eIF4G homolog PABP-interacting protein (PAIP) [48•].

The 5′- and 3′-UTRs, especially of some viral genes, can be fused to the mRNA of the protein of interest to enhance its translation rate *in vitro* (for examples see [49–53]). Some 5′-UTRs can enhance translation by functioning as an internal ribosome entry site (reviewed in [54]). As examples, the tobacco etch potyvirus and encephalomyocarditis virus 5′-UTRs were used to enhance translation of a reporter enzyme by promoting initiation in order to decrease dependance on the presence of a cap in the mRNA [18,55].

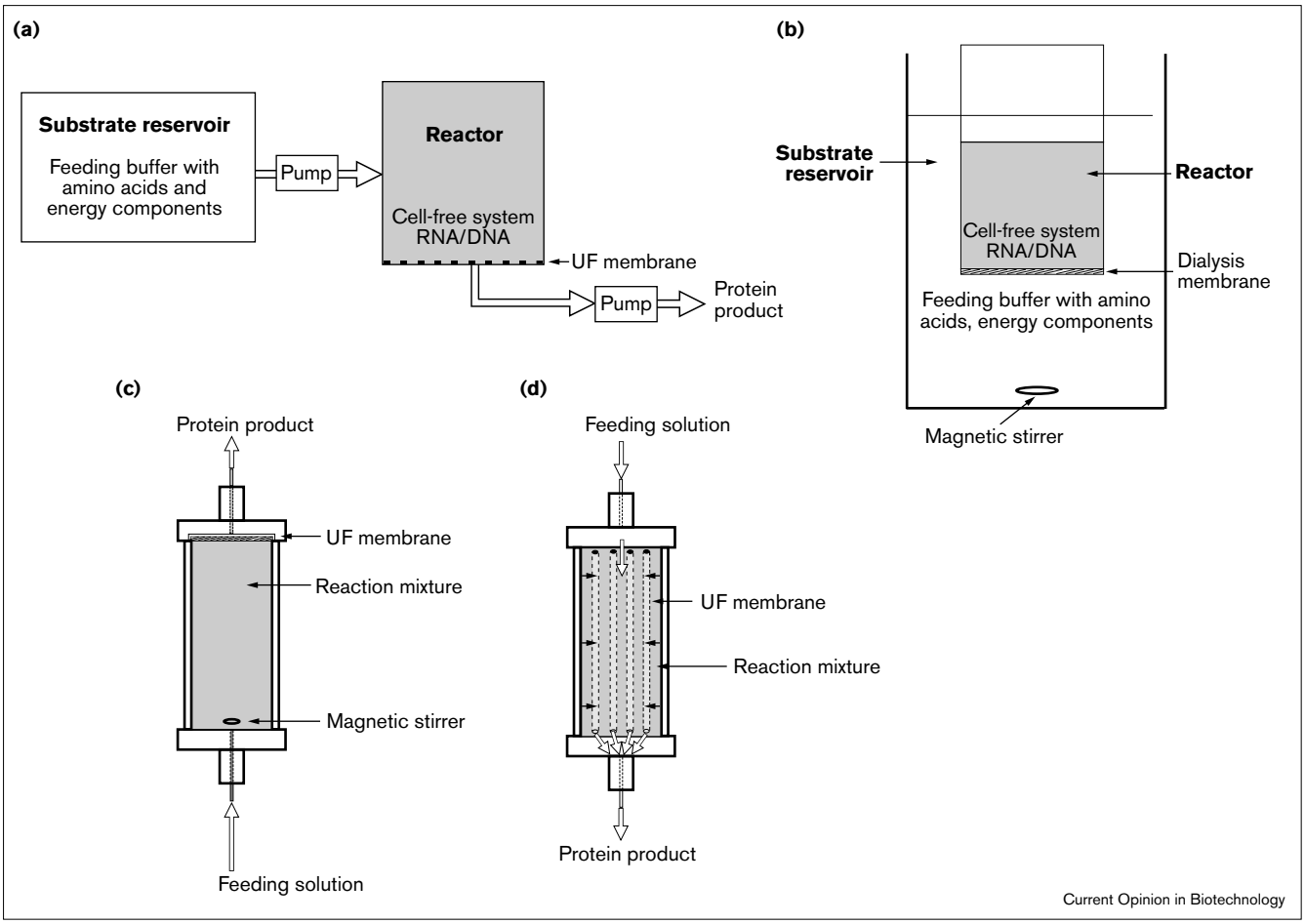
Less common translation systems

Other mammalian cell-free systems, such as from Ehrlich ascites cells and similar cell-types (e.g. human HeLa or mouse L-cells), have also been widely used, despite their comparatively low protein synthesis activity. Recently, an *in vitro* translation system from isolated tobacco chloroplasts was developed [56]. This system is strongly dependent on the exogenously added mRNA, produces discrete translational products and gives reproducible results, but also suffers from low protein yields. The extracts from *Saccharomyces cerevisiae* are now extensively used for studying the mechanisms of gene expression in yeast. *In vitro* translation in yeast is strongly dependent on a 5′-terminal cap and a 3′-poly(A) tail, but cap-independent translation can also be studied in the yeast cell-free system [57]. Using a yeast S30 extract, *in vitro* evidence was provided that the poly(A)-tail and PABPs mediate the recruitment of ribosomal subunits to the 5′-end of mRNA [58]; however, the high level of degradation activity and relatively low protein yield are limiting parameters of these systems for protein production. In summary, functional *in vitro* systems can in principle be prepared from any cell type, but many factors contribute to protein production efficiency.

Continuous-flow cell-free expression systems

The main obstacles for cell-free translation carried out in batch mode are the short duration of translational activity and the consequently low yield. As detailed above, the mRNA degradation and depletion of nucleotide triphosphates and the accumulation of their hydrolysates are the central molecular causes of low yield. The problem of how to achieve longer reaction times of cell-free expression was solved by Spirin and co-workers [59,60] several years ago by using a continuous-flow cell-free (CFCF) translation aparatus. The basic idea is to continuously supply energy components and amino acids (feeding solution) and to continuously remove small molecule by-products (mainly products of triphosphate hydrolysis) and the synthesized polypeptides. The protein bioreactor (Figure 1a) consists of a reservoir from which the feeding solution is continuously pumped through the reaction chamber, which contains all translation components. The products are continuously removed from the reaction chamber through an ultrafiltration membrane with a MW cut-off in the range of 10–300 kDa and collected by a fraction collector. This system can function for more than 20 hours and results in preparative protein expression of on average 0.1–0.3 mg protein ml^{-1} reaction volume, accumulated during 20–30 hours of synthesis. The template for translation in this system can either be mRNA directly [60–62], DNA transcribed by the endogenous bacterial RNA polymerase or by added phage RNA polymerase [21,29,60,63] or self-replicating RNA in the presence of Qβ replicase [64]. In

Figure 1



Overview of continuous flow systems. **(a)** Scheme of continous flow cell-free (CFCF) and **(b)** semicontinous flow cell-free (SFCF) protein synthesis systems. All components required for translation or coupled transcription/translation are inside the reactor. Amino acids and energy components are supplied in the feeding buffer from the substrate reservoir. The protein product and small molecular by-products, such as trinucleotide hydrolysates, are removed from the reactor either through the ultrafiltration (UF) membrane in (a) the CFCF system, or the hydrolysates get accumulated in the substrate reservoir in (b) the SFCF system, while the protein product can be retained by the dialysis membrane, depending on the pore size. The reactor needs to be stirred gently. **(c,d)** Schematic drawing of two alternative CFCF bioreactors. In (c), the ultrafiltration membrane is located at the top of the reaction chamber [63], and (d) shows a hollow fiber membrane reactor [69].

most cases, undoubtedly depending on the particular protein product, proteins produced in CFCF systems remain active even after prolonged incubation. For example, the CFCF synthesis of the cytokine interleukin-6 with the same specific activity as recombinant interleukin-6 obtained from bacteria (see Table 1) and with a purity of greater than 80% in the effluent was reported [62].

A number of laboratories attempting CFCF expressions have had difficulties, however, in establishing this complex system. The main problems are the RNA degradation when using bacterial extracts (even in the coupled transcription/translation mode), the low efficiency of initiation complex formation (which might cause leakage and, therefore, loss of some translation components by ultrafiltration), and clogging of the ultrafiltration membrane, resulting in a low rate of protein synthesis compared to the batch format. Also, the problem of reproducibility of the system needs to be solved.

Recently, several groups have reproduced and significantly improved this technology [5,12,65]. These studies provided modifications that might be critical, or at least can considerably enhance the performance and reproducibility of the system. Translation initiation is thought to be, in most cases, the rate limiting step in protein synthesis. In eukaryotes, the 5′-cap structure strongly stimulates the translation initiation events and increases mRNA stability, as detailed above, and the use of capped RNA should result in a higher rate of protein synthesis. For example, cytoplasmic aldolase from rice (MW of 40 kDa) was produced with the specific activity of the

Table 1

Summary of recent results on protein production in cell-free flow bioreactors.

Mode of translation	Cell-free system	Expressed protein	Time of synthesis (h)	Membrane	Produced protein (μg/ml)	Activity (U/mg)	Reference
CFCF translation	S23 wheat germ extract	DHFR	17	XM 50	27.4	nd (active)	[61]
		IL-6	50	XM 50	93	0.5×10^3	[62]
CFCF translation (capped mRNA)	S23 wheat germ extract	ALD	30	XM 50	255	12×10^3	[5]
CFCF coupled transcription/translation with SP6 RNA polymerase in the presence of cap-analogue	S23 wheat germ extract	ALD	30	XM 50	205	14×10^3	[5]
CFCF coupled transcription/translation with T7 RNA polymerase	*E. coli* S30 extract	CAT	20	YM 100	143	5.6×10^3	[12]
SFCF coupled transcription-translation with T7 RNA polymerase	*E. coli* S30 extract	CAT	14	Dialysis membrane (MW cut-off = 8–10 kD)	1200	nd (low activity)	[65]

ALD, rice cytoplasmic aldolase; CAT, chloramphenicol acetyltransferase; CFCF, continous-flow cell-free; DHFR, dihydrofolate reductase; IL-6, interleukin 6; nd, not determined; SFCF, semicontinuous-flow cell-free; XM and YM are trade names from AMICON.

native enzyme in a CFCF wheat germ cell-free translation system at 37°C, using capped RNA or a coupled transcription/translation system, presumably generating capped mRNA, both resulting in similar yields of about 6 μg ml^{-1} h^{-1} [5]. The incubation temperature in both cases was 37°C, which is unusually high for the wheat germ system, indicating that the stability of the capped mRNA was not significantly affected by increasing the incubation temperature.

The rate of feeding should be carefully optimized [12,66], and this appears to be important for the maintenance of high concentrations of triphosphates during the CFCF run [18]. It was shown that protein synthesis can stop if the biochemical energy level, defined by Atkinson [67] as ([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP]), declined to 0.85, and restarted when the energy level was raised to 1.0 by replenishment of ATP and GTP [18]. This suggests that the success of the CFCF system is mainly due to the constant maintenance of the high energy level and the low level of triphosphate hydrolysis products in the reaction mixture.

Thio-modified mRNA can also be used for long-time translation reactions. The higher translation activity of ATP-substituted thio-mRNA encoding dihydrofolate reductase compared with unsubstituted mRNA was shown for an *E. coli* CFCF translation system during six hours of translation [39].

The problem of continuous product removal from the protein bioreactor is still not fully solved. The use of ultrafiltration membranes has some limitations, because proteins may not pass through the membrane, or they may stick to the membrane. There is another way of carrying out prolonged expression, namely by using diffusion instead of pumping to supply substrates and remove by-products. This has been called a semicontinous flow cell-free system (SFCF) [65]. The reaction mixture is separated from the feeding solution by a dialysis membrane. Continuous supply of low MW substrates and removal of by-products, but not synthesized polypeptides, is achieved by in- and outward diffusion using dialysis membranes with small MW cut-offs (Figure 1b). The high accumulation of chloramphenicol acetyltransferase has been reported after 14 hours of a semicontinous flow cell-free system with an *E. coli* transcription/translation system [65] (Table 1); however, the synthesized protein has to be purified from the translation mixture. Similar strategies to those used in *in vivo* protein production have been considered, such as immobilized affinity ligands, to which the protein product will bind even directly in the reaction mixture. The synthesis of active dihydrofolate reductase was reported for a bacterial cell-free system in the presence of matrix-bound methotrexate, an affinity ligand [68]. Additionally, the use of the affinity ligand may stabilize the native structure of the protein during continued protein synthesis.

A third type of bioreactor for CFCF synthesis, termed a hollow fiber membrane reactor, was initially proposed by Spirin [60] and experimentally demonstrated by Suzuki and co-workers [69]. Here, the feeding solution enters via an ultrafiltration fiber coil in the chamber with the reaction mixture (Figure 1d). The advantage of this reactor is its large membrane filtration area compared to the reactor

volume. The production of functional luciferase in this bioreactor during a 20 hour run was reported, but the yield was low and further development is required [69].

Cotranslational folding and folding catalysts

One of the central problems in biotechnological applications of cell-free translation is whether the protein will fold correctly. If the protein folds post-translationally, this would be identical to refolding *in vitro*, except that the starting conformation is not a random coil, solubilized by a denaturant solution, but kept soluble by binding to chaperones. In cotranslational folding, however, the nascent protein would sequentially attain its structure while one end of the chain is still attached to the ribosome, which may or may not facilitate folding by forcing an order of events on the folding of protein domains. In both co- and post-translational folding, molecular chaperones may block off-pathways to aggregation, and catalysts, such as prolyl *cis-trans* isomerases, may catalyze individual folding steps. Normally, no disulfide-forming or disulfide-isomerization catalysts are present in the cytoplasm but these can, however, be added exogeneously to the cell-free system (see below).

Recently, the question of cotranslational folding was reviewed independently by two groups [70,71•]. Both emphasize the observation that cotranslational folding is an essential characteristic of the folding pathway of many proteins of prokaryotes and eukaryotes alike, and its occurrence may even depend on the exact conditions. The hypothesis of some authors that cotranslational folding on prokaryotic ribosomes is generally a rare event [72] appears to be in contradiction with a number of published results on folding in cell-free systems, which include reports on cotranslational protein folding in prokaryotic *in vitro* systems (see [70,71•] and references therein).

The folding of rhodanese in an *E. coli* extract [73] as well as the formation of enzymatically active luciferase in a wheat germ extract [74,75], both while still attached to the ribosome, has been demonstrated in constructs where the protein was carboxy-terminally extended by at least 25 additional amino acid residues to allow the folded enzyme to protrude from the ribosome. The binding of co-factors, ligands or substrates to nascent polypeptides can also occur cotranslationally. Ribosome-bound α-globin fragments longer than 86 amino acid residues are fully capable of heme binding [76]. Finally, ribosome display experiments [23••,77••] suggest that a eukaryotic protein can fold cotranslationally and remain attached to either eukaryotic or prokaryotic ribosomes, if a long enough carboxy-terminal tether sequence ensures that the entire protein is outside of the ribosomal complex and can fold there. It should be noted, however, that in such experiments the release of the protein from the ribosome is artificially retarded. The folded state can, therefore, have been reached after the protein would have normally been released. It has been shown recently that free luciferase displays an increased enzymatic activity, however, compared to luciferase still attached to the ribosome via a tether sequence [78]. Although the authors ascribe this observation to putative additional conformational rearrangements after protein release from the ribosome, it might also be due to the fact that some shorter protein species would attain the native structure only once they are dissociated from the ribosome.

Molecular chaperones and folding catalysts, such as prolyl *cis-trans* isomerase, are known to be important factors in cotranslational protein folding (for review see [71•]). Several chaperones have been shown to interact with the nascent polypeptide (see [71•,79] and references therein). These include the trigger factor (a peptidyl-prolyl *cis-trans* isomerase), DnaJ/DnaK/GrpE and GroEL/GroES in prokaryotic cells and members of the hsp60 and 70 family in eukaryotic cells.

In eukaryotic cells, disulfide formation occurs in the endoplasmic reticulum while the nascent protein is being transported through the membrane [80]. In *E. coli*, disulfide formation, catalyzed by the DsbABCD proteins [81]; occurs in the periplasm, and both cotranslational and post-translational mechanisms have been described [82]. In order to produce preparative amounts of disulfide-containing, functional antibodies in an *E. coli* cell-free translation system, the factors contributing to efficient folding and proper disulfide bond formation were identified [83•]. It was suggested that chaperones, mostly DnaK and DnaJ, influence the amount of 'soluble' single-chain antibody fragments (scFv) in an *E. coli* S30 cell-free system, but do not affect the amount of 'functional' proteins, indicating that there are soluble, misfolded species. Eukaryotic protein disulfide isomerase as an efficient disulfide isomerization catalyst, added cotranslationally in the cell-free system, dramatically increased the amount of functional antibodies. Evidence was provided that the isomerization reaction, and not the net disulfide bond formation, is crucial in the *in vitro* folding of scFv fragments. The use of proper redox conditions and the addition of folding catalysts can, therefore, have a significant effect on the preparative production of disulfide-containing biologically active proteins *in vitro*.

It has long been suspected that the ribosomes themselves might be involved in protein folding. It has now been found experimentally that the large prokaryotic and eukaryotic ribosomal subunits can have chaperone-like activity *in vitro* in that they increase the *in vitro* folding yield of several substrate proteins [84,85]. As such an activity might in principle be unspecific and not of biological relevance, it was of great interest to test whether specific antibiotics that block particular sites on the ribosome might inhibit this activity. Indeed, such an inhibition was found [86], and it was deduced that the 23S and 28S rRNA, particularly the domain V of 23S rRNA, seem to be involved. Furthermore, a chaperone-like activity in elongation factor EF-Tu was

found during *in vitro* refolding experiments [87]. Unfortunately, no genetic experiments to test the role of these activities *in vivo* are possible, as these components are, of course, all essential. To study these questions *in vitro* an *E. coli* system deficient in molecular chaperones has been described recently [88].

Applications

A clear advantage of *in vitro* translation compared to *in vivo* expression is the relative ease with which single components of the system, such as a specific tRNA, can be altered or exchanged. In addition, compared to *in vivo* experiments, the simultaneous expression of several proteins and the study of their interaction is relatively straightforward. On the other hand, the impact of this technology is still limited by comparatively low yields and is currently used only for analytical purposes.

Incorporation of unnatural amino acids and selective labelling

The possible extension of the genetic code by using unnatural amino acids is one of the most exciting features of *in vitro* systems. In the past, protocols for the alternative acylation of tRNAs have been published, where a suppressor tRNA, which binds to a stop codon, was charged chemically and enzymatically with an unnatural amino acid (reviewed in [89]). By introducing a suppressable termination codon in the protein sequence and by translating the sequence in an *in vitro* system, supplemented with the charged suppressor tRNA, an efficient incorporation of the new amino acid residue at the engineered position was achieved. Of course, at the very best, stoichiometric amounts of a chemically charged tRNA can be converted to proteins and, usually, very much smaller amounts are obtained. Nevertheless, this methodology has been used to address questions of protein stability, signal transduction and enzyme mechanisms with unnatural amino acids. Recently, this strategy was used to probe the mechanism of aspartate aminotransferase with unnatural amino acids [90••], an experiment which is so far impossible by *in vivo* protein engineering. In addition, the use of suppressor tRNAs with unnatural amino acids further enhances the possibilities of directed evolution *in vitro* (see next section).

Instead of using unnatural amino acids, Wemmer and coworkers [91] demonstrated that the incorporation of a particular ^{13}C-labelled residue, encoded as described above by a suppressor tRNA, made it possible to follow this amino acid upon denaturation and refolding of the protein by NMR spectroscopy. In order to make large proteins available for NMR studies, a segment-selective labelling method has been proposed [92]. The idea is based on using translation mixes depleted of either one amino acid and/or its tRNAs and/or its tRNA-synthetase and consists of three steps. The template RNA is coupled to a column so that the translation reaction mixture can be easily exchanged. First, the unlabelled amino-terminal region is synthesized up to the first codon without matching tRNA. The ribosomes pause, and the translation mix is exchanged against a mix containing ^{13}C-labelled amino acids, but now lacking a different amino acid, tRNA or tRNA-synthetase. Translation resumes, thereby labelling the region until ribosomes encounter the first codon without corresponding tRNA. In the last step, the carboxy-terminal portion is synthesized without label, and the protein is released from the ribosome. This method produces a protein in which a specific region contains ^{13}C-labelled amino acids to facilitate NMR studies; however, to date the technique is suffering from low protein yields, as it can, at the very best, produce protein stoichiometric to the immobilized mRNA.

It should be noted that the use of selective labeling or unnatural amino acids by *in vitro* translation is receiving serious competition from the chemical synthesis of whole proteins. By using chemical segment condensation of unprotected peptides, a new realm of protein synthesis is accessible [93••]. Furthermore, the discovery of inteins (protein segments that get spliced out at the protein level, rather than at the RNA level) has lead to some exciting developments in biochemical segment condensation [94]. It will have to be seen how *in vitro* translation and chemical synthesis of large amounts of complex proteins with unnatural amino acids will compare, but the advent of segment condensation chemistry may put total chemical synthesis currently in the lead.

Fast production and analysis of protein mutants

The direct expression of PCR products, carrying engineered or random mutations, with *in vitro* systems [23••,31•,33] has been demonstrated. Here, the sequence pool from a PCR reaction can be directly used for *in vitro* expression. The effects observed with the produced mutant protein can be quite safely attributed to the engineered mutation (provided the primers are correct), as PCR errors will be unimportant because a pool of sequences is analyzed. During the cloning of PCR products for *in vivo* expression, however, the descendants of a single molecule are analyzed, always requiring DNA sequencing as a control. Using this mutagenesis technique the entire binding region of an antibody has been rapidly scanned and mapped (termed scanning saturation mutagenesis [31•]). Nevertheless, a quantitative analysis (where all mutations have been analyzed by dissociation constants or, in the case of an enzyme, kinetic parameters) has not yet been reported. Like many other *in vitro* techniques, scanning saturation mutagenesis is an ideal candidate for automation. The limitation of this technology is reached, however, when certain analyses need large amounts of protein or the protein to be purified from the translation reaction.

Eukaryotic *in vitro* translation systems offer a very simple possibility to produce and study carboxy-terminally truncated proteins to investigate, for example, domain

functions or define epitopes. In contrast to *E. coli* extracts, these systems lack a strong endogenous RNase and RNase H activity. The addition of DNA primers complementary to the mRNA directly or after a prior incubation with exogenous RNase H provokes ribosome stalling at the heteroduplex, followed by the release of the truncated polypeptide from the ribosome [95]. Alternatively, genes coding for defined truncated proteins can nowadays be obtained very easily by using PCR and, following transcription, the mRNA can subsequently be used for producing the protein *in vitro* by direct expression [75]. On the other hand, *in vitro* expression with eukaryotic translation systems has also been used to uncover disease-causing nonsense mutations that lead to premature translation termination. This technique, termed protein truncation test, is based on isolating RNA from blood or tissue samples, producing cDNA and, after PCR, expressing the protein *in vitro* and analyzing its size [96].

Complex membrane assemblies

The rabbit reticulocyte lysate *in vitro* translation system has been used together with microsomal membranes to study the biosynthesis of membrane proteins [97], protein translocation and post-translational modifications, such as signal peptide cleavage or core glycosylation. Furthermore, the chaperones resident in the endoplasmic reticulum (such as protein disulfide isomerase and calnexin) can be used to facilitate the folding of secreted proteins.

Recently, several groups have reported the production of functional lipoproteins [98] and membrane proteins, such as the T-cell receptor–CD3 complex [99••], inositol triphosphate receptors [100] and ion channels [101•]. Functionality was monitored either by specific assembly or oligomerization or by a functional assay.

Two examples will pinpoint the degree of complexity that can be achieved with *in vitro* systems. First, by adjusting the redox conditions Huppa and Ploegh [99••] could successfully cotranslate the six chains of the T-cell receptor–CD3 complex together with CD4 and the two chains of the corresponding MHC. Interestingly, the complex only formed when all chains were expressed simultaneously, as one CD3 subunit only oxidized correctly when cotranslated with the mRNA of the other CD3 elements. All nine chains integrated into the microsomal membranes, and in the presence of the appropriate peptide ligand assembled specifically to form a complex. The subunit-specific assembly of the TCR was demonstrated by immunoprecipitation with antibodies against single chains of the complex. The authors concluded from these experiments that glycosylation and microsome-embedded chaperones were crucial to this process.

The second example describes the cell-free assembly of functional gap junction membrane channels [101•]. Connexins, translated in rabbit reticulocyte lysate, integrated into the microsomal membrane and assembled to form hexameric gap junction membrane channels. These complexes were subsequently reconstituted in lipid bilayers by fusion with the microsomal membranes. Single channel patch clamp recordings could then demonstrate their functionality. The advantage of this method compared to *in vivo* expression in complex heterologous systems, such as oocytes or tissue culture cells, may be the simpler interpretation of the results, as fewer components are present.

Making virus *in vitro*

The demonstration of the *de novo* synthesis of infectious poliovirus in a cell-free extract from uninfected HeLa cells by Molla and co-workers [102] gave an efficient tool to study different stages of phage and viral morphogenesis. About 10 hours were necessary for the synthesis of infectious particles, and the *in vitro* reactions support all stages of the process, from the translation of the viral RNA to polyprotein processing, RNA replication, and assembly of infectious virus particles. Since then, the cell-free assembly of viral capsids has been reported for Mason-Pfitzer monkey virus [55] and HIV type I [103,104], two retroviruses which follow different types of capsid assembly in the host cell. *In vitro* assembly was also demonstrated for MS2 phage [105].

Replication-competent poliovirus RNA polymerase functions in replication, in the genetic recombination of poliovirus RNA, as well as in the generation of infective poliovirus recombinants. It was studied in a cell-free system derived from HeLa-cells [106,107]. Thus, cell-free systems can also be useful tools to study the mechanism of viral RNA replication and evolution of RNA viruses.

Another multifunctional polymerase from duck hepatitis B virus was produced in reticulocyte lysate by *in vitro* transcription/translation [108,109]. This enzyme, which includes the activities of DNA polymerase, reverse transcriptase and RNase H, was difficult or impossible to express *in vivo* as an active enzyme. *In vitro* translation is not a miracle cure, however, as the production of active human hepatitis B virus polymerase is still difficult *in vitro* as well as *in vivo*.

Recently, the assembly of immature retrovirus capsids from Gag monomers has been reproduced *in vitro* to study the process of virus production. The formation of a type D retrovirus capsid, Mason-Pfitzer monkey virus, in a reticulocyte coupled transcription/translation system mimics the assembly into procapsids in the host cell cytoplasm [55,110]. The formation of immature HIV-1 capsid, which *in vivo* requires plasma membrane targeting, was also shown to occur in cell-free reaction systems, prepared either from reticulocyte lysate [103] or wheat germ extract [104].

An *E. coli* extract programmed with phage MS2 RNA was used to produce MS2 infectious units which were able to infect *E. coli* F^+ cells [105]. It was suggested, however, that the nature of this infectivity is the formation of minimal

infectious units, consisting of RNA and maturation proteins. Unlike normal MS2 phages, the particles produced *in vitro* are very sensitive to RNase or protease treatment.

In vitro selection from large libraries

The principle

All selection techniques currently used for screening libraries of peptides or proteins make use of living cells for producing the polypeptides. Examples are phage display, selectively infectious phages (SIP), peptides on plasmids, *E. coli* and yeast surface display, the two-hybrid system and others [111]. As a consequence, their initial library size is limited by the efficiency of transformation. The more complex the design of the library, the smaller the fraction of clones which can actually be screened. In an evolutionary strategy, selected molecules must be randomized after each round. Because of the labor involved in transforming large libraries, evolution over many generations is very cumbersome with *in vivo* methods. The only way to gain fast and convenient access to library sizes of 10^{12} or higher is the development of alternative selection tools that are independent of transformation.

Any selection technique is based on the coupling of genotype and phenotype: the amplifiable genomic information (DNA or RNA) must be physically linked to the selectable entity (the peptide or protein). As outlined in Figure 2, two different approaches have been published which make such coupling possible in an *in vitro* translation set-up. The first principle is referred to as ribosome display [23••] in this review, although other names such as polysome display [112,113], polysome selection [24] and ARM selection [77••] have been used. The key to success for selecting whole proteins was to find conditions so that the polypeptide folds correctly but does not leave the ribosome, the mRNA does not leave the ribosome and the polypeptide recognizes a specific ligand thereby coupling genotype and phenotype. The method is based on the possibility of expressing peptides and proteins from mRNA lacking a stop codon and the direct use of the ternary complexes, consisting of polypeptide, the ribosome and the encoding mRNA, for affinity enrichment (Figure 2a). The absence of a stop codon prevents release from the ribosome. That such ternary complexes might form and can be trapped is suggested from observations made by several groups [114–117], who all found that specific mRNA could be enriched by precipitating the ribosomes with antibodies against the protein product. Before the advent of molecular cloning, this technique was very important for isolating the mRNA corresponding to a particular protein. After affinity selection the RNA is recovered, reverse transcribed and PCR-amplified. This PCR product can then serve as a template for the next selection round. The most exciting aspect of this technology is that when a polymerase without proofreading activity, such as Taq, is used for PCR, mutations are introduced into the template such that an evolution of the protein can be achieved by going through several rounds [23••,118•]. The selection of functional proteins using this principle necessitated a thorough optimization of mRNA stability and cotranslational protein folding [23••].

The second selection principle has been published under the name '*in vitro* virus' [119•] or 'RNA–peptide fusion' [120••] and is directly related to ribosome display, but uses a puromycin-tagged RNA. Puromycin is chemically coupled to DNA, which in turn is ligated to the RNA encoding a peptide or protein library. At the end of translation the coupled antibiotic serves as an acceptor for the nascent polypeptide chain emerging from the ribosome (Figure 2a). The peptide–nucleic acid fusion is thus isolated. Instead of a ribosome-mediated complex, the result of this technique is a direct and chemically more robust linkage of a peptidyl chain and the encoding nucleic acid. Under physiological conditions and at low temperature, however, both the ribosome-mediated and the fusion complex seem to be comparably stable. The preparation of the puromycin–mRNA fusion, which has to be repeatedly constructed for each selection cycle, is clearly a time-limiting factor, but the fusion complexes may be used under very harsh selection conditions (high temperature, high concentration of denaturants) as long as the RNA does not hydrolyze.

Apart from the independence of transformation, another advantageous feature of the two *in vitro* selection techniques is the ease with which the genetic information of all selected molecules can be recovered, as they do not have to be eluted. Instead of requiring selective and complex elution strategies, ribosome disruption or direct RT-PCR allow for the selection of very tight and even covalent binders (Figure 2b).

Applications

Following the classical work on ribosome-bound polypeptides from the 1970's and 1980's [114–117], ribosome display was suggested a number of years ago [P1], yet without giving any experimental details. The first experimental demonstration came only recently for a selection of peptide ligands using an *E. coli* extract [112,113]. In the past year, the use of a wheat germ lysate has also been reported for peptide selection [24]. The selection of functional antibody fragments was reported using a translation system, optimized for the yield of ternary complexes and allowing disulfide formation [23••]. A diluted, antigen-binding antibody fragment was selected 10^8-fold over several rounds from a mixture with non-binders. The most exciting observation was that all selected molecules had acquired several mutations due to PCR errors without losing functionality. More recently, this experimental setup has been used to select antibodies from a murine library, and it was shown that affinity maturation occurs during the selection due to the combined effect of PCR errors and selection. An scFv fragment with a dissociation constant of about 10^{-11} M was obtained [118•]. Selection of binding antibody fragments from a large, synthetic library and a thorough analysis of the effect of the chosen *in vitro* translation

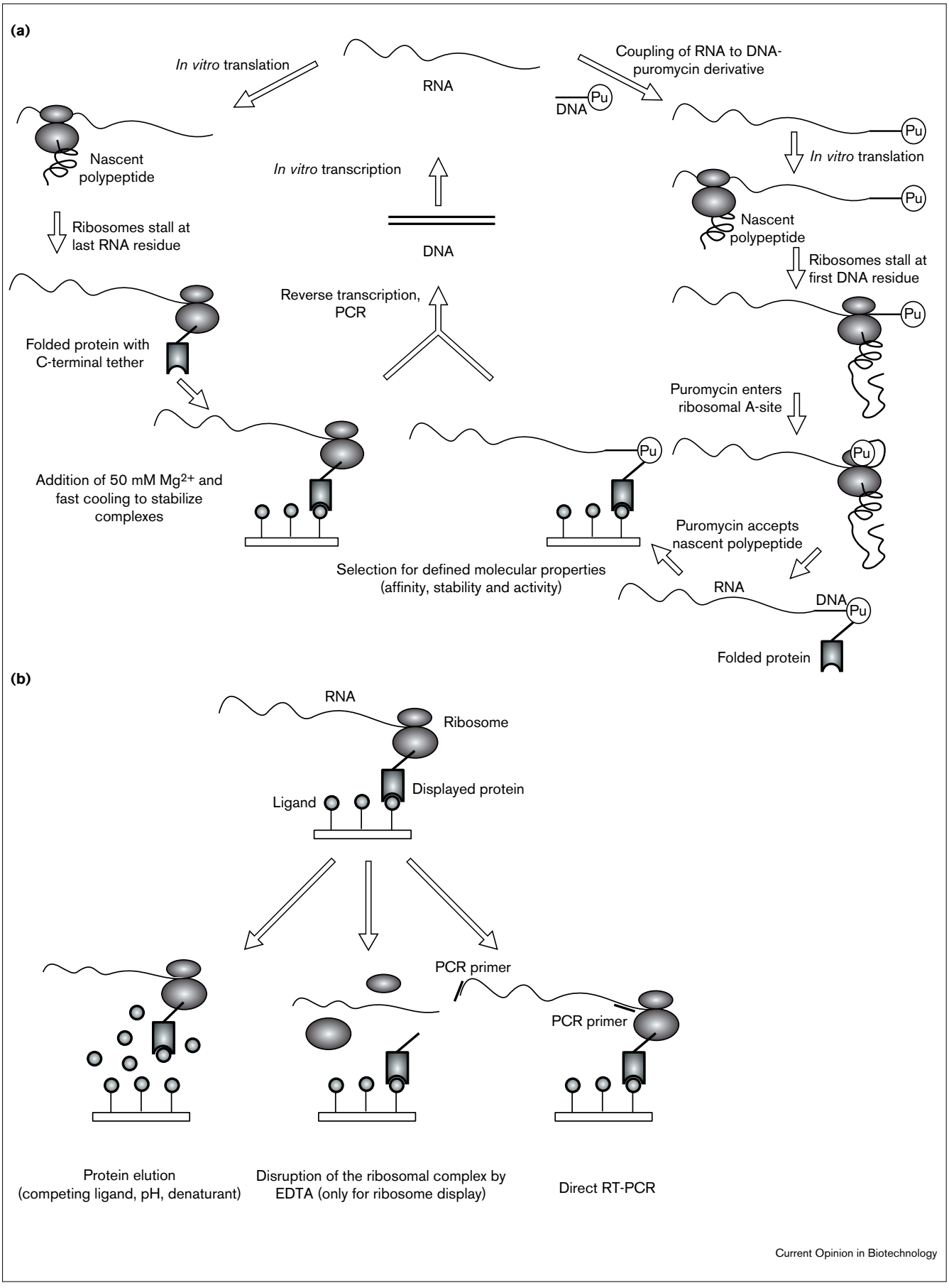

(a)
In vitro translation
RNA
Coupling of RNA to DNA-puromycin derivative
DNA
Pu
Nascent polypeptide
In vitro transcription
In vitro translation
Ribosomes stall at last RNA residue
DNA
Nascent polypeptide
Ribosomes stall at first DNA residue
Reverse transcription, PCR
Folded protein with C-terminal tether
Puromycin enters ribosomal A-site
Addition of 50 mM Mg2+ and fast cooling to stabilize complexes
Puromycin accepts nascent polypeptide
Selection for defined molecular properties (affinity, stability and activity)
RNA
DNA
Folded protein
(b)
RNA
Ribosome
Displayed protein
Ligand
PCR primer
PCR primer
Protein elution (competing ligand, pH, denaturant)
Disruption of the ribosomal complex by EDTA (only for ribosome display)
Direct RT-PCR
Current Opinion in Biotechnology

Figure 2

Schematic representation of the two current *in vitro* selection techniques **(a)** and their elution strategies **(b)**. In (a), ribosome display is described on the left hand side, while the puromycin (pu)-based method is outlined in the right hand panel. Both techniques use as starting point mRNA, encoding a library of peptides or proteins but containing no stop codon. Here, the two methods are compared for displaying a single-chain antibody fragment (which has not yet been shown for the puromycin-based method). There are three important differences. First, in the puromycin-based method the protein can fold in principle after its release from the ribosome (e.g. if it is synthesized without the carboxy-terminal tether), whereas in ribosome display it has to fold while still attached to the ribosomal complex. Second, the puromycin-based method allows for a direct linkage between RNA and protein. Third, ribosome display consists of far fewer steps and is faster to perform. **(b)** Selected molecules can be recovered by traditional protein elution, as in phage display (addition of soluble antigen, low or high pH, high salt concentration, denaturants). *In vitro* techniques offer two alternative elution strategies, EDTA-elution (only possible for ribosome display) and direct RT-PCR (for ribosome display and the puromycin-based method) to quantitatively recover selected RNA molecules. These methods, therefore, allow selection for high-affinity and even covalent binders.

system on the selection process are in progress (L Jermutus *et al*., unpublished data).

The use of commercial rabbit reticulocyte lysate was reported for the enrichment of a three-domain antibody-construct from a binary mixture [77••]. However, translation and selection were carried out in the presence of 2 mM dithiothreitol, which does not appear to be of general applicability to antibodies as many would not fold under reducing conditions [83•]. Furthermore, no assay of the functionality of the enriched protein, such as by radio-immunoassay or ELISA, was reported.

For the puromycin-based method, the existence of the fusion molecule [119•] and the selection of binding peptides [120••] from a doped random library have been shown. Both experiments used a rabbit reticulocyte lysate for *in vitro* translation, but the selection of folded proteins has not been reported yet. Currently, it is difficult to assess the pros and cons of the puromycin-based technique compared to ribosome display (see above).

In vitro selection techniques appear to be not only suited for affinity selection from large libraries but also for directed evolution. Because the PCR amplification can be easily coupled with random mutagenesis or PCR-mediated recombination, several cycles of selection and mutagenesis/recombination are possible in a short time, as no cloning steps are involved. Furthermore, it is not necessary to define the sequence stretches to be randomized (such as complementary-determining regions or frameworks in the case of scFvs) by first creating a library with degenerate primers. Instead, random mutagenesis on the entire coding region and appropriate selection conditions can evolve molecules with the desired properties. The challenge for the future will be to clearly describe the selective pressure for enriching molecules with defined properties, such as affinity or stability.

Conclusions

In vitro translation, when carried out under conditions optimizing protein folding, is a powerful technology for contributing to many scientific questions. PCR has been the key ingredient in many new exciting developments, such as rapid scanning mutagenesis or the translation-based selection and screening technologies. Whereas progress in large scale translation has been made on many fronts, it is more gradual, and no serious competition to *in vivo* methods is expected at least for the immediate future. Progress in analytical methods, however, may make it possible to obtain answers to many questions with the amounts of materials conveniently accessible from current cell-free translation protocols. The combination of fast mutant analysis or the use of unnatural amino acids with the functional expression of toxic or complex proteins constitutes a promising alternative to *in vivo* expression. The set-up of cell-free translation is fast and in most cases simple, an important aspect considering the number of protein sequences without attributed function which are continuously being discovered in various genome sequencing projects. Finally, it appears that the cell-free selection methods are an extremely powerful tool, and will bring the directed evolution of proteins (evolution implying gradual improvement over many generations) to reality shortly.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Spirin AS: **Ribosome preparation and cell-free protein synthesis.** In *The Ribosome: Structure, Function and Evolution*, vol 2. Edited by Hill EH, Dahlberg A, Garrot RA, Moore PB, Schlessinger D, Warner JR. Washington DC: American Society for Microbiology; 1990:56-70.

2. Stiege W, Erdmann VA: **The potentials of the *in vitro* biosynthesis system.** *J Biotechnol* 1995, **41**:81-90.

3. Zubay G: ***In vitro* synthesis of protein in microbial systems.** *Annu Rev Genet* 1973, **7**:267-287.

4. Roberts BE, Patterson BM: **Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ.** *Proc Natl Acad Sci USA* 1973, **70**:2330-2334.

5. Tulin EE, Ken-Ichi T, Shin-Ichiro E: **Continously coupled transcription-translation system for the production of rice cytoplasmic aldolase.** *Biotechnol Bioeng* 1995, **45**:511-516.

6. Pelham HRB, Jackson RJ: **An efficient mRNA-dependent translation system from reticulocyte lysates.** *Eur J Biochem* 1976, **67**:247-256.

7. • Yao SL, Shen XC, Suzuki E: **Biochemical energy consumption by wheat germ extract during cell-free protein synthesis.** *J Ferment Bioeng* 1997, **84**:7-13.

The report shows that the sudden cessation of protein synthesis in a wheat germ cell-free system is mainly caused by the rapid dephosphorylation of ATP and GTP. The authors demonstrate that energy replenishment is necessary for continuous protein synthesis.

8. Matveev SV, Vinokurov LM, Shaloiko LA, Matveeva EA, Alakhov YB: **Effect of the ATP level on the overall protein biosynthesis rate in a wheat germ cell-free system.** *Biochim Biophys Acta* 1996, **1293**:207-212.

9. Mosca JD, Wu JM, Suhadolnik PJ: **Restoration of protein synthesis in lysed rabbit reticulocytes by the enzymatic removal of adenosine 5′-monophosphate with either AMP deaminase or AMP nucleosidase.** *Biochemistry* 1983, **22**:346-354.

10. Hucul JA, Henshaw EC, Young DA: **Nucleoside diphosphate regulation of overall rates of protein biosynthesis acting on the level of initiation.** *J Biol Chem* 1985, **260**:15585-15591.

11. Felipo V, Grisolia S: **5′-adenosin monophosphate inhibits ternary complex formation by rat liver eIF2.** *Biochem Biophys Res Commun* 1987, **146**:1079-1083.

12. Kitaoka Y, Nishimura N, Niwano M: **Cooperativity of stabilized mRNA and enhanced translational activity in the cell-free system.** *J Biotechnol* 1996, **48**:1-8.

13. Nakano H, Matsuda K, Okumura R, Yamane T: **Accumulation of translational inhibitor during multi-hour cell-free protein synthesis reaction using rabbit reticulocyte lysate.** *J Ferment Bioeng* 1997, **83**:470-473.

14. de Haro C, de Herreros AG, Ochoa S: **Activation of the heme-stabilized translational inhibitor of reticulocyte lysates by calcium ions and phospholipid.** *Proc Natl Acad Sci USA* 1983, **80**:6843-6847.

15. Ryabova LA, Vinokurov LM, Shekhovtsova EA, Alakhov YB, Spirin AS: **Acetyl phosphate as an energy source for bacterial cell-free translation systems.** *Anal Biochem* 1995, **226**:184-186.

16. Kawarasaki Y, Nakano H, Yamane T: **Prolonged cell-free protein synthesis in a batch system using wheat germ extract.** *Biosci Biotechnol Biochem* 1994, **58**:1911-1913.

17. Yao S, Shen XC, Terada S, Suzuki E: **A novel method of high yield cell-free protein synthesis.** *J Ferment Bioeng* 1997, **84**:548-552.

18. Kawarasaki Y, Kawai T, Nakano H, Yamane T: **A long-lived batch reaction system of cell-free protein synthesis.** *Anal Biochem* 1995, **226**:320-324.

19. Nakano H, Tananka T, Kawarasaki Y, Yamane T: **An increased rate of cell-free protein synthesis by condensing wheat germ extract with ultrafiltration membranes.** *Biosci Biotechnol Biochem* 1994, **58**:631-634.

20. Kim DM, Kigawa T, Chen CY, Yokoyama S: **A highly efficient cell-free protein synthesis system from *Escherichia coli*.** *Eur J Biochem* 1996, **239**:881-886.

21. Kudlicki W, Kramer G, Hardesty B: **A highly efficient cell-free protein synthesis system from *Escherichia coli*.** *Eur J Biochem* 1992, **239**:881-886.

22. Gurevich VV: **Use of bacteriophage RNA polymerase in RNA synthesis.** *Methods Enzymol* 1996, **275**:382-397.

23. •• Hanes J, Plückthun A: ***In vitro* selection and evolution of functional proteins by using ribosome display.** *Proc Natl Acad Sci USA* 1997, **94**:4937-4942.

First experimental demonstration of selection of a folded protein for ligand binding using ribosome display. Selected antigen-binding antibody fragments were shown to have evolved over many cycles.

24. Gersuk GM, Corey MJ, Corey E, Stray JE, Kawasaki GH, Vessella RL: **High-affinity peptide ligands to prostate-specific antigen identified by polysome selection.** *Biochem Biophys Res Commun* 1997, **232**:578-582.

25. Chen HZ, Zubay G: **Prokaryotic coupled transcription-translation.** *Methods Enzymol* 1983, **101**:674-690.

26. Nevin DE, Pratt JM: **A coupled *in vitro* transcription-translation system for the exclusive synthesis of polypeptides from the T7 promotor.** *FEBS Lett* 1991, **291**:259-263.

27. Köhrer C, Mayer C, Grübner P, Piendl W: **Use of T7 RNA polymerase in an optimized *Escherichia coli* coupled *in vitro* transcription-translation system. Application in regulatory studies and expression of long transcription units.** *Eur J Biochem* 1996, **236**:234-239.

28. Craig D, Howell MT, Gibbs CL, Hunt T, Jackson RJ: **Plasmid cDNA-directed synthesis in a coupled eukaryotic *in vitro* transcription-translation system.** *Nucleic Acids Res* 1992, **20**:4987-4995.

29. Baranov VI, Spirin AS: **Gene expression in cell-free systems on preparative scale.** *Methods Enzymol* 1993, **217**:123-142.

30. Jacques N, Dreyfus M: **Translation initiation in *Escherichia coli*: old and new questions.** *Mol Microbiol* 1990, **4**:1063-1067.

31. • Burks EA, Chen G, Georgiou G, Iverson BL: ***In vitro* scanning saturation mutagenesis of an antibody binding pocket.** *Proc Natl Acad Sci USA* 1997, **94**:412-417.

By combining PCR-mutagenesis and direct *in vitro* expression of the resulting PCR products the authors could rapidly analyze the influence of each amino acid in an antibody binding pocket.

32. Lesley SA, Brow MA, Burgess RR: **Use of *in vitro* protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies.** *J Biol Chem* 1991, **266**:2632-2638.

33. Martemyanov KA, Spirin AS, Gudkov AT: **Direct expression of PCR products in a cell-free transcription/translation system: synthesis of antibacterial peptide cecropin.** *FEBS Lett* 1997, **414**:268-270.

34. Chetverin AB, Spirin AS: **RQ RNA vectors: prospects for cell-free gene amplification, expression and cloning.** *Prog Nucleic Acid Res Mol Biol* 1995, **51**:225-270.

35. Kozak M: **Selection of initiation sites by eukaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin.** *Nucleic Acids Res* 1984, **12**:3873-3893.

36. Kozak M: **Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6.** *EMBO J* 1997, **16**:2482-2492.

37. Sprengart ML, Porter AG: **Functional importance of RNA interactions in selection of translation initiation codons.** *Mol Microbiol* 1997, **24**:19-28.

38. Ovodov SY, Alakhov YB: **mRNA acetylated at 2′-OH groups of ribose residues is functionally active in the cell-free translation system from wheat embryos.** *FEBS Lett* 1990, **270**:111-114.

39. Tohda H, Chikazumi N, Ueda T, Nishikawa K, Watanabe K: **Efficient expression of *E. coli* dihydrofolate reductase gene by an *in vitro* translation system using phosphorothioate mRNA.** *J Biotechnol* 1994, **34**:61-69.

40. Ueda T, Tohda H, Chikazumi N, Eckstein F, Watanabe K: **Phosphorothioate-containing RNAs show mRNA activity in the prokaryotic translation system *in vitro*.** *Nucleic Acids Res* 1991, **19**:547-552.

41. Ehretsmann CP, Carpousis AJ, Kirsch HM: **mRNA degradation in prokaryotes.** *FASEB J* 1992, **6**:3186-3191.

42. Arnold TE, Belasco JG: **mRNA stabilization by the *omp*A 5′ untranslated region: two protective elements hinder distinct pathways for mRNA degradation.** *RNA* 1998, **4**:319-330.

43. Ross J: **mRNA stability in mammalian cells.** *Microbiol Rev* 1996, **59**:423-450.

44. DeMaria CT, Sun Y, Wagner BJ, Brewer G: **Structural determinants in AUF1 required for high affinity binding to A+U-rich elements.** *J Biol Chem* 1997, **272**:27635-27643.

45. Wickens M, Anderson P, Jackson RJ: **Life and death in the cytoplasm: messages from the 3′ end.** *Curr Opin Gen Dev* 1997, **7**:220-232.

46. •• Tarun SZ, Wells SE, Deardorff JA, Sachs AB: **Translational initiation factor eIF-G mediates *in vitro* poly(A) tail-dependent translation.** *Proc Natl Acad Sci USA* 1997, **94**:9046-9051.

The authors provide evidence that yeast translation factor eIF-4G features a binding site for poly(A)-binding protein Pab1p. This leads to an interaction

between eIF-4G, a subunit of cap-associated initiation factor eIF-4F at the 5′ end of the mRNA and poly(A)–Pab1p at the 3′ ends of the mRNA.

47. •• Le H, Tanguay RL, Balasta ML, Wei CC, Browning KS, Metz AM, Goss DJ, Gallie DR: **Translation initiation factors eIF-iso4G and eIF-4B interact with the poly(A)-binding protein and increase its RNA binding activity.** *J Biol Chem* 1997, **272**:16247-16255.
This report demonstrates the interaction between cap-associated initiation factor eIF-iso4F and eIF-4B with the poly(A)-binding protein (PABP) in plants. eIF-iso4G, the subunit of eIF-iso4F, was found to be involved in the binding of PABP. A similar mechanism in yeast and plants seems to participate in 3′-UTR regulation of mRNA initiation and turnover.

48. • Craig AWB, Haghighat A, Yu ATK, Sonenberg A: **Interaction of polyadenylate-binding protein with the eF4G homologue PAIP enhances translation.** *Nature* 1998, **392**:520-522.
The authors propose that the interaction between mRNA ends in mammals is mediated by a novel poly(A)-binding protein, PAIP, which is homologous to the central portion of eF-4G. PAIP also interacts with another subunit of cap-binding factor eIF-4F, ATP-dependent RNA helicase, eIF-4A.

49. Jobling SA, Gehrke L: **Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence.** *Nature* 1987, **325**:622-625.

50. Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA: **The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*.** *Nucleic Acids Res* 1987, **15**:3257-3273.

51. Danthinne X, Seurinck J, Meulewater F, van Montagu M, Cornelissen M: **The 3′ untranslated region of satellite tobacco necrosis virus RNA stimulates translation *in vitro*.** *Mol Cell Biol* 1993, **13**:3340-3349.

52. Timmer RT, Benkowski LA, Schodin D, Lax SR, Metz AM, Ravel JM, Browning KS: **The 5′ and 3′ untranslated regions of satellite tobacco necrosis virus RNA affect translational efficiency and dependence on a 5′ cap structure.** *Mol Cell Biol* 1993, **13**:3340-3349.

53. Zeyenko VV, Ryabova LA, Gallie DR, Spirin AS: **Enhancing effect of the 3′-untranslated region of tobacco mosaic virus RNA on protein synthesis *in vitro*.** *FEBS Lett* 1994, **354**:271-273.

54. Sonnenberg N: **mRNA translation: influence of the 5′ and 3′ untranslated regions.** *Curr Opin Genet Dev* 1994, **4**:310-315.

55. Sakalian M, Parker SD, Weldon RA, Hunter E: **Synthesis and assembly of retrovirus Gag precursors into immature capsids *in vitro*.** *J Virol* 1996, **70**:3706-3715.

56. Hirose T, Sugiura M: ***Cis*-acting element and *trans*-acting factors for accurate translation of chloroplast *psbA* mRNAs: development of an *in vitro* translation system from tobacco chloroplasts.** *EMBO J* 1996, **15**:1687-1695.

57. Iizuka N, Sarnow P: **Translation-competent extracts from *Saccharomyces cerevisiae*: effects of L-A RNA, 5′ cap, 3′ poly(A) tail on translational efficiency of mRNAs.** *Methods* 1997, **11**:353-360.

58. Tarun SZ, Sachs AB: **A common function for mRNA 5′ and 3′ ends in translation initiation in yeast.** *Genes Dev* 1995, **9**:2997-3007.

59. Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB: **A continuous cell-free translation system capable of producing polypeptides in high yield.** *Science* 1988, **242**:1162-1164.

60. Spirin AS: **Cell-free protein bioreactor.** In *Frontiers in Bioprocessing II*. Edited by Todd P, Skidar SK, Bier M. Washington DC: American Chemical Society; 1991:31-43.

61. Endo Y, Oka T, Ogata K, Natori Y: **Production of dihydrofolate reductase by an improved continous flow cell-free translation system using wheat germ extract.** *Tokshima J Exp Med* 1993, **40**:13-17.

62. Volyanik EV, Dalley A, McKay IA, Leigh I, Williams NS, Bustin SA: **Synthesis of preparative amounts of biologically active interleukin-6 using a continous-flow cell-free translation system.** *Anal Biochem* 1993, **214**:289-294.

63. Kigawa T, Yokoyama S: **A continous cell-free protein synthesis system for coupled transcription-translation.** *J Biochem* 1991, **110**:166-168.

64. Ryabova L, Volyanik E, Kurnasov O, Spirin AS, Wu Y, Kramer FR: **Coupled replication-translation of amplifiable messenger RNA.** *J Biol Chem* 1994, **269**:1501-1505.

65. Kim DM, Choi CY: **A semicontinous prokaryotic coupled transcription/translation system using a dialysis membrane.** *Biotechnol Prog* 1996, **12**:645-649.

66. Baranov VI, Morozov IY, Ortlepp SA, Spirin AS: **Gene expression in a cell-free system on the preparative scale.** *Gene* 1989, **84**:463-466.

67. Atkinson DE: **The energy charge of the adenylate pool as a regulatory parameter: interaction with feedback modifiers.** *Biochemistry* 1968, **7**:4030-4034.

68. Marszal E, Scouten WH: **Dihydrofolate reductase synthesis in the presence of immobilized methotrexate. An approach to a continuous cell-free protein synthesis system.** *J Mol Recognit* 1996, **9**:543-548.

69. Yamamoto YI, Nagahori H, Yao S, Zhang ST, Suzuki E: **Hollow fiber reactor for continous flow cell-free protein production.** *J Chem Eng Japan* 1996, **6**:1047-1050.

70. Kolb VA, Makayev EV, Kommer A, Spirin AS: **Cotranslational folding of proteins.** *Biochem Cell Biol* 1995, **73**:1217-1220.

71. • Fedorov AN, Baldwin TO: **Cotranslational protein folding.** *J Biol Chem* 1997, **272**:32715-32718.
Concise review about the early events of *in vivo* protein folding. The authors summarize the role of chaperones and folding kinetics during protein biosynthesis.

72. Netzer WJ, Hartl FU: **Recombination of protein domains facilitated by co-translational folding in eucaryotes.** *Nature* 1997, **388**:343-349.

73. Kudlicki W, Odom OW, Kramer G, Hardesty B, Merrill GA, Horowitz PM: **The importance of the N-terminal segment for DnaJ-mediated folding of rhodanese while bound to ribosomes as peptidyl-tRNA.** *J Biol Chem* 1995, **18**:10650-10657.

74. Kolb VA, Makeyev EV, Spirin AS: **Folding of firefly luciferase during translation in a cell-free system.** *EMBO J* 1994, **13**:3631-3637.

75. Makeyev EV, Kolb VA, Spirin AS: **Enzymatic activity of the ribosome-bound nascent polypeptide.** *FEBS Lett* 1996, **378**:166-170.

76. Komar AA, Kommer A, Krasheninnikov IA, Spirin AS: **Cotranslational folding of globin.** *J Biol Chem* 1997, **272**:10646-10651.

77. •• He M, Taussig MJ: **Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites.** *Nucleic Acids Res* 1997, **25**:5132-5134.
Ribosome display of an antibody fragment with a commercial eukaryotic transcription/translation system.

78. Yang F, Jing GZ, Zhou JM, Zheng YZ: **Free luciferase may acquire a more favorable conformation than ribosome-associated luciferase for its activity expression.** *FEBS Lett* 1997, **417**:329-332.

79. Rassow J, Pfanner N: **Protein biogenesis: chaperones for nascent polypeptides.** *Curr Biol* 1996, **6**:115-118.

80. Bergman LW, Kuehl WM: **Formation of intermolecular disulfide bonds on nascent immunoglobulin polypeptides.** *J Biol Chem* 1979, **254**:5690-5694.

81. Bardwell JC: **Building bridges: disulphide bond formation in the cell.** *Mol Microbiol* 1994, **14**:199-205.

82. Randall LL, Josefsson LG, Hardy SJ: **Processing of exported proteins in *Escherichia coli*.** *Biochem Soc Trans* 1980, **8**:413-415.

83. • Ryabova LA, Desplancq D, Spirin AS, Plückthun A: **Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones.** *Nat Biotechnol* 1997, **15**:79-84.
The authors describe a thorough optimization of cell-free production of functional antibody fragments (scFvs). Protein disulfide isomerase and the molecular chaperones DnaJ and DnaK have the most prominent effect on the amount of functional and soluble scFv, respectively.

84. Das B, Chattopadhyay S, Bega AK, Dasgupta C: ***In vitro* protein folding by ribosomes from *Escherichia coli*, wheat germ and rat liver: the role of 50S particle and its 23S RNA.** *Eur J Biochem* 1996, **235**:613-621.

85. Kudlicki W, Coffman A, Kramer G, Hardesty B: **Ribosomes and ribosomal RNA as chaperones for folding of proteins.** *Fold Des* 1997, **2**:101-108.

86. Chattopadhyay A, Das B, Dasgupta C: **Reactivation of denatured proteins by 23S ribosomal RNA: role of domain V.** *Proc Natl Acad Sci USA* 1996, **93**:8284-8287.

87. Kudlicki W, Coffman A, Kramer G, Hardesty B: **Renaturation of rhodanese by translational elongation factor (EF) Tu.** *J Biol Chem* 1997, **272**:32206-32210.

88. Kramer G, Zhang T, Kudliski W, Hardesty B: **Preparation and application of chaperone-deficient *Escherichia coli* cell-free translation system.** *Methods Enzymol* 1998, **290**:18-26.

89. Cornish VW, Schultz PG: **Site-directed mutagenesis with an expanded genetic code.** *Annu Rev Biophys Biomol Struct* 1995, **24**:435-462.

90. •• Park Y, Luo J, Schultz PG, Kirsch JF: **Noncoded amino acid replacement probes of the aspartate aminotransferase mechanism.** *Biochemistry* 1997, **36**:10517-10525.
By incorporating unnatural amino acids into the enzyme using *in vitro* translation the authors could decipher key elements of the enzymatic mechanism.

91. Ellman JA, Volkman BF, Mendel D, Schultz PG, Wemmer DE: **Site-specific isotopic labeling of proteins for NMR studies.** *J Am Chem Soc* 1992, **114**:7959-7961.

92. Pavlov MY, Freistoffer DV, Ehrenberg M: **Synthesis of region-labelled proteins for NMR studies by *in vitro* translation of column-coupled mRNAs.** *Biochimie* 1997, **79**:415-422.

93. •• Muir TW, Dawson PE, Kent SB: **Protein synthesis by chemical ligation of unprotected peptides in aqueous solution.** *Methods Enzymol* 1997, **289**:266-298.
Review of the protocols for the chemical ligation reaction yielding a normal peptide bond, which makes the synthesis of large proteins possible. As the peptide segments are made by normal solid phase synthesis, unnatural amino acids can be encoded anywhere.

94. Cooper AA, Stevens TH: **Protein splicing: self-splicing of genetically mobile elements at the protein level.** *Trends Biochem Sci* 1995, **20**:351-356.

95. Haeuptle MT, Frank R, Dobberstein B: **Translation arrest by oligodeoxynucleotides complemetary to mRNA coding sequences yields polypeptides of predetermined length.** *Nucleic Acids Res* 1986, **14**:1427-1447.

96. van Essen AJ, Kneppers AL, van der Hout AH, Scheffer H, Ginjaa IB, ten Kate LP, van Ommen GJ, Buys CH, Bakker E: **The clinical and molecular genetic approach to Duchenne and Becker muscular dystrophy: an updated protocol.** *J Methods Genet* 1997, **34**:805-812.

97. Walter P, Blobel G: **Preparation of microsomal membranes for cotranslational protein translocation.** *Methods Enzymol* 1983, **96**:84-93.

98. Rusinol AE, Jamil H, Vance JE: ***In vitro* reconstitution of assembly of apolipoprotein B48-conatining lipoproteins.** *J Biol Chem* 1997, **272**:8019-8025.

99. •• Huppa JB, Ploegh HL: ***In vitro* translation and assembly of a complete T cell receptor-CD3 complex.** *J Exp Med* 1997, **186**:393-403.
A remarkable example of the complexity which can be reached with *in vitro* systems. The chains for the complete T-cell receptor–CD3 complex together with the chains for its corresponding MHC complex were simultaneously translated and assembled.

100. Joseph SK, Boehning D, Pierson S, Niccitta CV: **Membrane insertion, glycosylation, and oligomerization of inositol triphosphate receptors in a cell-free translation system.** *J Biol Chem* 1997, **272**:1579-1588.

101. • Falk MM, Buehler LK, Kumar NM, Gilula NB: **Cell-free synthesis and assembly of connexins into functional gap junction membrane channels.** *EMBO J* 1997, **16**:2703-2716.
The authors describe the functional reconstitution of a multimeric membrane transporter *in vitro*. The membrane channels were reconstituted into synthetic lipid bilayers for functionality tests.

102. Molla A, Paul AV, Wimmer E: **Cell-free, *de novo* synthesis of poliovirus.** *Science* 1991, **254**:1647-1651.

103. Spearman P, Ratner L: **Human immunodeficiency virus type 1 capsid formation in reticulocyte lysates.** *J Virol* 1996, **70**:8187-8194.

104. Lingappa JR, Hill RL, Wong ML, Ramanujan SH: **A multistep, ATP-dependent pathway for assembly of human immunodeficiency virus capsids in a cell-free system.** *J Cell Biol* 1997, **136**:567-681.

105. Katanaev VL, Spirin AS, Reuss M, Siemann M: **Formation of bacteriophage MS2 infectious units in a cell-free translation system.** *FEBS Lett* 1996, **397**:143-148.

106. Tang RS, Barton DJ, Flanegan JB, Kirkegaard K: **Poliovirus RNA recombination in cell-free extracts.** *RNA* 1997, **3**:624-633.

107. Duggal R, Cuconati A, Gromeier M, Wimmer E: **Poliovirus RNA recombination in cell-free extracts.** *Proc Natl Acad Sci USA* 1997, **94**:13786-13791.

108. Howe AY, Elliott JF, Tyrrell DL: **Duck hepatitis B virus polymerase produced by *in vitro* transcription and translation possesses DNA polymerase and reverse transcriptase activities.** *Biochem Biophys Res Commun* 1992, **189**:1170-1176.

109. Wang GH, Seeger C: **The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis.** *Cell* 1992, **71**:663-670.

110. Weldon RA, Parker WB, Sakalian M, Hunter E: **Type D retrovirus capsid assembly and release are active events requiring ATP.** *J Virol* 1998, **72**:3098-3106.

111. Phizicky EM, Fields S: **Protein-protein interactions: methods for detection and analysis.** *Microbiol Rev* 1995, **59**:94-123.

112. Mattheakis LC, Bhatt RR, Dower WJ: **An *in vitro* polysome display system for identifying ligands from very large peptide libraries.** *Proc Natl Acad Sci USA* 1994, **91**:9022-9026.

113. Mattheakis LC, Dias JM, Dower WJ: **Cell-free synthesis of peptide libraries displayed on polysomes.** *Methods Enzymol* 1996, **267**:195-207.

114. Schechter I: **Biologically and chemically pure mRNA coding for a mouse immunoglobulin L-chain prepared with the aid of antibodies and immobilized oligothymidine.** *Proc Natl Acad Sci USA* 1973, **70**:2256-2260.

115. Payvar F, Schimke RT: **Improvements in immunoprecipitation of specific messenger RNA.** *Eur J Biochem* 1979, **101**:271-282.

116. Korman AJ, Knudsen PJ, Kaufman JF, Strominger JL: **cDNA clones for the heavy chain of HLA-DR antigens obtained after immunoprecipitation of polysomes by monoclonal antibody.** *Proc Natl Acad Sci USA* 1982, **79**:1844-1848.

117. Kraus JP, Rosenberg LE: **Purification of low-abundance messenger RNAs from rat liver by polysome immunoadsorption.** *Proc Natl Acad Sci USA* 1982, **79**:4015-4019.

118. • Hanes J, Jermutus L, Bornhauser-Weber S, Bosshard HR, Plückthun A: **Ribosome display selects and evolves high-affinity binding antibodies from murine libraries.** *Proc Natl Acad Sci USA* 1998, in press.
The first demonstration that a diverse protein library can be screened with ribosome display. The authors observed a 'built-in' affinity maturation occuring simultaneously to the selection.

119. • Nemoto N, Miyamoto-Sato E, Husimi Y, Yanagawa H: ***In vitro* virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome *in vitro*.** *FEBS Lett* 1997, **414**:405-408.
A protocol for the puromycin-mediated fusion of mRNA to its encoding protein.

120. •• Roberts RW, Szostak JW: **RNA-peptide fusions for the *in vitro* selection of peptides and proteins.** *Proc Natl Acad Sci USA* 1997, **94**:12297-12302.
The authors demonstrate in a model system that the puromycin-based *in vitro* selection technique is able to enrich binding ligands out of a doped peptide library.

Patent

P1. Kawasaki G: **Screening randomized peptides and proteins with polysomes**. *PCT International Application* 1991, WO 91/05058.